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## FACTORS IN THE METABOLISM OF LACTOSE.

### I. THE DISPOSAL OF INTRAVENOUSLY ADMINISTERED GALACTOSE IN THE RABBIT.\*

BY RAPLH C. CORLEY.

(From the Department of Bio-Chemistry of the School of Medicine, Tulane University, New Orleans.).

(Received for publication, April 29, 1927.)

The interest pertaining to lactose, because it is the carbohydrate in the diet of the nursling, is magnified by virtue of a number of other more or less related considerations. Half of its molecule is composed of galactose, to which is attributed the importance of serving as a source of certain essential tissue constituents, particularly those of the nervous system. Is it to be concluded that this explains the reason for the presence of this unique carbohydrate in the diet of the developing infant, or are there inherent in the lactose molecule other properties of value? Once this latter question might have been answered in the affirmative because of the inclusion of glucose in the molecule, but there now seems little doubt that the accompanying glucose facilitates the metabolism of the galactose (Folin and Berglund, 1922; Bodansky, 1923).

As one factor involved in this effect, there is to be considered the report of Cori (1925-26), that when glucose and galactose were absorbed together, the total amount absorbed was not greater than if either alone was present. In commenting on the results of Folin and Berglund, Benedict and Osterberg (1923) were inclined to feel, "that 'dilution' of the galactose with the glucose may be regarded as an adequate explanation of the facts unless experimental evidence is offered which disproves this view." Bodansky (1923), while admitting that such a factor could not be omitted from consideration, believed that the absorption rate could not be the important factor, because levulose in contradistinction to glucose had such a slight if any effect on galactose hyperglycemia. In this regard, however, it is well to remember that the effect of levulose on galactose absorption has not been quantitatively determined, that levulose has been said to be more slowly absorbed than glucose or galactose (Cori, 1925), and that it is rather hazardous to draw conclusions based on the reducing power of a medley of reducing

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substances in the blood. Further than this absorptive effect, the mechanism of the relationship between the metabolism of these two aldohexoses is exceedingly obscure.

While the presence of glucose causes the tolerance for lactose to exceed that for galactose, the relationship is reversed when the two substances are introduced through any channel other than the digestive tract. Although apparently the disposal of galactose is little dependent on its method of entering the blood stream, the absence of a circulating lactase makes difficult the utilization of any milk sugar administered parenterally. As pointed out by Folin and Berglund, further evidence is required as to the fate of lactose under these conditions, since conclusions based on the determination of the extent of the melituria following injection of this sugar are not unobjectionable. It is, however, possible to say that any considerable utilization of lactose is dependent on an initial hydrolysis in the alimentary tract, and that any unsplit lactose that may be absorbed when the digestive ability is exceeded is excreted largely in the urine. In essence then, the problem of the further fate of lactose is that of the fate of the products of hydrolysis. There has been presented no evidence that the glucose resulting from lactose digestion is not further metabolized readily. Yet, mention must be made of the suggestive results of Reinhold and Karr (1927) who found a striking difference in the blood sugar curves after the administration of lactose and after mixtures of glucose and galactose, and were led to consider the possibility of a difference between the *in vivo* and the acid hydrolysis products of lactose. Pending definite conclusions along these lines, it is scarcely unjustifiable to assume that the metabolism of lactose and galactose are intimately related, and that information about the latter may be applied directly in the interpretation of the fate of the former.

Considerable interest attaches itself to the metabolism of galactose. It comprises half of the carbohydrate of the diet of the young of mammals; it appears in the diet of older individuals particularly so far as there are consumed milk, certain milk products, or a number of other substances containing galactose; it is employed in building up various tissues; and it readily yields energy in the body.

That there is some more or less intimate connection between glucose and galactose would seem to be indicated since galactose is converted to glycogen, may be converted to glucose in the diabetic organism, is probably formed from glucose in the lactating mammary gland, and may function similarly to glucose in a number of other physiological situations. Galactose is reported to relieve insulin intoxication (Moschini, 1924; Voegtlin *et al.*, 1925; Noble and Macleod, 1923; Cassidy *et al.*, 1926; Herring, Irvine, and Macleod, 1924) to induce the shivering complex in hypoglycemia (Cassidy *et al.*, 1926) and to be somewhat beneficial in the hypoglycemia following extirpation of the liver although this effect is slight and transitory (Mann and Magath, 1922).

Elaboration of the methods of disposal of galactose might be expected to be pertinent to the question of the fates of lactose and of glucose, and furthermore, since glucose and galactose differ but slightly in structural

configuration, the comparison of their fates may yield evidence as to the manner that stereochemical alterations affect physiological utilization. To make more complete the data for speculation along these lines, it would be valuable to have more information about *d*-mannose, which differs from glucose and galactose merely in the space relationships of one carbon atom.

The paths of disposal of any substance gaining entrance to the blood stream are various. It may be excreted, stored, or burned as such, or it may be converted to another substance or substances, which then may be excreted, stored, or burned. The rate of disposal will be dependent on the interrelationships existing between such of these factors as are operative. Experimental variations of any one of them should probably furnish evidence in regard to its importance. While the alimentary tract probably does excrete sugars, the main excretory channel of such substances is by way of the kidneys. Experimental nephritis and diabetes may be considered as representing decreased and increased renal permeability respectively. The liver is of great importance in the metabolism of certain sugars, functioning as a storehouse of reserve carbohydrates, and probably as a place of transformation of certain other substances to glucose as well (Bollman, Mann, and Magath, 1926). It should be of interest to learn the influence of liver poisoning on the utilization of a variety of carbohydrates. Insulin furnishes a means for producing a pronounced effect on sugar metabolism, at least so far as the glucose-glycogen system is concerned. Insulin, then might be expected to affect the fate of a foreign carbohydrate to an extent dependent upon the relation between the metabolism of this substance and that of the quantitatively most important sugars of the body. Folin and Berglund (1922) have expressed the belief that absorption of sugars by the tissues is the primary method of the body in reducing their concentration in the blood. Hines and Leese (1926-27) have reported that pituitrin decreased the rate of absorption of glucose from the blood by the tissues, although they suggested that circulatory changes might have been an important factor.

Blood analyses have been of great value in studying the problems of metabolism, and are particularly effective in following the rate of disposal of different substances entering the blood stream. The study of galactose has undoubtedly been handicapped by the lack of a method for its determination in small amounts. A great deal of the information about this compound has been obtained by studying the concentration of the total blood sugar. While probably most investigators have found that galactose caused a marked hyperglycemia, all reports have not been in agreement (MacLean and de Wesselow, 1921; Folin and Berglund, 1922; Bodansky, 1923; Schätti, 1923; London *et al.*, 1924; Goldblatt, 1925; Barrenscheen *et al.*, 1926). Several possibilities may account for this divergence of results. The nutritive condition of the organism has a marked effect on galactose disposal (Reinhold and Karr), perhaps due to the influence of glucose, at least in part. Evaluation of the published figures is difficult because they are for total reduction of the blood. The objection to such a procedure lies in the fact that changes in the total blood sugar represent the algebraic

summation of the changes that have taken place in the reducing constituents present and can only be assumed as due to any individual substance. Consequently hyperglycemia may be due either to large amounts of circulating galactose or merely to an increased concentration of glucose. Analogously, normal blood sugar values may either mean that very small or negligible amounts of galactose are present in the blood or that the galactose concentration is balanced by a decreased glucose concentration (Barrenschéen, 1926), and in this regard, it is noteworthy that since galactose has a lower reducing power than glucose such a contingency is the more readily conceivable. Furthermore, might not the flooding of the organism with a carbohydrate, utilizable at least to a certain degree, cause a decreased requirement for glucose resulting in a true hypoglycemia such as Folin and Berglund (1922) discuss as occurring during prolonged sugar absorption or even as due to fat ingestion?

Exemplifications of both of the above possibilities are in the findings of Isaac (1920). He has reported figures in which after the administration of fructose, when the blood sugar concentration rose from 96 to 104 mg. per 100 cc. the glucose concentration dropped to 68 mg. and the fructose had a value of 36 mg. per 100 cc. In yet another case, the levulose content of the blood was 23 mg. per 100 cc. although the total blood sugar dropped from 104 to 98 mg. per 100 cc. On the other hand, a rise in blood sugar from 94 to 104 mg. was caused by an increase in the glucose to 102 mg. and a fructose value of 2 mg. per 100 cc. Since the glucose and fructose concentrations in the results of Isaac are based on calculations from polariscopic and reduction determinations, they probably are not unobjectionable, for as Brasch (1908) has indicated in regard to a similar procedure, small variations, probably within the limits of experimental error, may make great differences in the calculated results. These results of Isaac may not be typical but they do emphasize the danger of drawing conclusions from the total reduction of the blood when several carbohydrates are or may be present.

The present communication reports the rate of removal of intravenously injected galactose in the normal rabbit, and in the rabbit under conditions of tartrate and uranium nephritis, of chloroform and phosphorus poisoning, and of phlorhizin diabetes. Direct introduction into the blood obviates the complications due to absorptive interrelationships.

#### EXPERIMENTAL.

The details of this series of experiments may be summarized as follows: The galactose dissolved in water was injected into the marginal ear vein of a rabbit that had been fasting for 24 to 48 hours. Male rabbits were employed except where indicated other-

wise. The blood samples (2 to 3 cc.) were collected at intervals from the other ear, using potassium oxalate as an anticoagulant, and analyzed for non-protein nitrogen, blood sugar, and reducing power after fermentation. The proteins were precipitated and the non-protein nitrogen determined according to the Folin-Wu procedure, and the reducing power of the filtrate determined by the Shaffer-Hartmann (1920-21) method, the results being expressed in terms of glucose. The galactose employed (Eastman Kodak Company preparation) had about 80 per cent of the reducing power of glucose which corresponds with results that have been reported by others (Greenwald, Samet, and Gross, 1924-25).

The method previously described for the determination of pentoses in the blood (Corley, 1926) is in general applicable to the estimation of those carbohydrates that are resistant to fermentation by yeast. As admitted at that time, such a method is open to a number of obvious objections. The fermentation itself may introduce errors since the yeast may contain reducing substances, may form reducing substances during fermentation, may fail to destroy completely the glucose of the blood, or may change the reducing properties of substances other than glucose in the blood. However, since under similar conditions, the reducing power of the blood after fermentation with yeast is small and fairly constant, no real difficulty arises. It is scarcely unjustifiable to assume that in the presence of galactose, the difference from the usual unfermented reducing power of the blood furnishes a reliable index of the amount of galactose present. A further objection is to be found in the fact that yeast probably destroys galactose to a certain extent. It is frankly admitted then that the figures for unfermented reduction in this paper are not to be considered as the true residual reducing power of the blood, and furthermore that this method is probably inadequate to detect small quantities of circulating galactose.

#### *Fermentation of Blood Sugar.*

1 cc. of whole blood was incubated an hour at 37°C. with 0.5 cc. of a yeast suspension made by suspending in 40 cc. of 0.9 per cent sodium chloride solution a cake of Fleischmann's compressed yeast, from which most of the starch had been removed by cen-

trifugation. The proteins were removed by the Folin-Wu method and the filtrate analyzed for residual reduction. As much as 5.0 mg. of glucose per cc. could be removed almost completely (95 to 100 per cent), while the recovery of added galactose was from 90 to 104 per cent, the low results having been obtained with small amounts of galactose (0.18 mg. per cc.).

Duggan and Scott (1926) have stated that there is a lack of oxidizing power of the Shaffer-Hartmann reagent, but that when reduction did take place the results were consistent. Since satisfactory agreement has been found with the figures of Duggan and Scott, and those obtained with low concentrations in this study, all calculations have been made from their table.

#### *Urine Analysis.*

The urinary excretion of galactose has been estimated by determining the unfermented reduction. The figures thus obtained are to be considered merely as an index of the maximum amount of galactose present, since small amounts of other non-fermenting reducing substances are continuously present in the urine of rabbits. Proteins present during nephritis were removed by heat coagulation. The galactose in the urine (*i.e.* the total unfermented reduction) was determined by the Shaffer-Hartmann micro method after fermentation with a yeast suspension for 1 hour at 37°C. The urine was so diluted that 5 cc. would contain an amount of sugar that was within the limits of the reagent. 20 cc. of this diluted urine after fermentation with 2 cc. of the yeast suspension were centrifuged and the supernatant liquid analyzed 94 to 98 per cent of added glucose and only 1 to 6 per cent of added galactose were destroyed under these conditions.

#### *Results.*

Table I presents typical results obtained with normal rabbits following the intravenous injection of galactose in amounts varying from 0.5 to 3.0 gm. The residual reduction returned to normal in 1 hour after the administration of 0.5 gm., in 2 hours after 1 gm., in 3 hours after 2 gm., and in practically 3 hours after 3 gm. The amount of galactose excreted in the urine increased with increasing dosage, the relation seeming to be of a higher function than linear

proportionality, although an attempt to indicate an exact mathematical relationship is unwarranted.

It is felt that one possible criterion of the rate of disposal of galactose is the value of the residual reduction at the hour preceded-

TABLE I.  
*Effect of Galactose on Blood Sugar.*

Rabbit No.	Weight.	Injected.		Reduction in blood per 100 cc.					Unfermented reduction.	
		Galactose.	Water.		Before.	After.	1 hr.	2 hrs.		3 hrs.
	kg.	gm.	cc.		mg.	mg.	mg.	mg.	mg.	mg.
32	1.5	0.5	8	Total.	91.8	157.2	101.8	99.0		
				Unfermented.	25.6	79.2	36.0	36.0		63
6	1.5	1.0	10	Total.	84.4	234.0	120.8	101.8		
				Unfermented.	34.6	165.0	48.0	30.0		120
31	1.9	1.0	16	Total.	110.0	218.6	120.8	101.8		
				Unfermented.	30.0	136.4	53.6	30.0		132
39	2.1	1.0	10	Unfermented.	94.2	190.0	113.0	101.8		
				Total.	28.6	123.4	56.6	30.0		104
40	1.9	1.0	10	Unfermented.	89.4	205.6	114.4	96.6		
				Total.	34.6	139.0	61.6	31.6		94
21	2.0	2.0	18	Unfermented.	89.4	336.6	208.2	139.0	101.8	
				Total.	30.0	263.6	131.2	69.0	33.0	370
20	2.2	2.0	18	Unfermented.	87.0	322.2	170.2	120.8	110.2	
				Total.	31.4	226.4	101.8	49.0	30.0	418
25	1.8	3.0	23	Unfermented.	87.0	365.4	229.0	146.8	131.2	
				Total.	27.0	281.4	139.0	84.4	36.0	532
9	2.1	3.0	20	Unfermented.	96.6	438.0	271.2	165.0	128.6	
				Total.	30.0	351.0	180.4	79.2	30.0	826
35*	1.5	1.0	10	Unfermented.	87.0	231.4	110.2	94.2	99.0	
				Total.	24.0	157.2	42.0	25.6	24.0	153
36*	1.5	1.0	10	Unfermented.	96.6	192.6	136.4	107.4	91.8	
				Total.	27.0	162.4	71.0	39.0	25.6	175

\* Female rabbit.

ing the return to the normal value. In fifteen experiments, following the injection of 1 gm. of galactose, the residual reduction at the end of 1 hour was between 48 and 62 mg. per 100 cc. There was no correlation observed between this value (or any other relationship considered) and the time of fasting (above 24 hours),



TABLE II.

*Effect of Tartrate Nephritis on Blood Sugar with Administration of Galactose.*

Rabbit 18, 1.9 kilos; white male.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
1926		mg.	mg.	mg.	mg.	
Dec. 7	5.10 p.m.					1.5 gm. tartaric acid in molecular $\text{Na}_2\text{CO}_3$ .
" 8	Before.	98.9	71.0	30.0		1 gm. galactose in 15 cc. water at 10.10 a.m.
" 8	10.13 a.m.		231.4	157.2		
" 8	11.13 "		126.0	61.6		
" 8	12.13 p.m.		74.2	30.0		
" 8	1.13 "		96.6	31.4	0	5 cc. urine.
" 12						Rabbit dead.

TABLE III.

*Effect of Uranium Nephritis on Blood Sugar with Administration of Galactose.*

Rabbit 25, 1.7 kilos; gray male.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
1926		mg.	mg.	mg.	mg.	
Dec. 12	4.40 p.m.					6 mg. uranium acetate in 10 cc. water.
" 13	Before.	47.9	74.0	25.6		1 gm. galactose in 15 cc. water at 10.55 a.m.
" 13	10.58 a.m.		241.0	133.8		
" 13	11.56 "		126.0	52.0		
" 13	12.55 p.m.		96.6	30.0		
" 13	2.23 "		113.0	25.6	147	Total reduction 160 mg.
" 14	10.00 a.m.				75	" " 268 "
" 21						Rabbit dead.

TABLE IV.

*Effect of Phlorhizin Poisoning on Blood Sugar with Administration of Galactose.*

Rabbit 23, 1.5 kilos; brown male.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
1926		mg.	mg.	mg.	mg.	
Dec. 12	4.30 p.m.					0.7 gm. phlorhizin in 10 cc. olive oil.
" 13	Before.	61.7	96.6	27.0		1 gm. galactose in 15 cc. water at 10.49 a.m.
" 13	10.52 a.m.		273.8	135.4		
" 13	11.53 "		146.8	60.0		
" 13	12.50 p.m.		120.8	30.0		
" 13	2.19 "		126.0	25.2	333	Total reduction 443 mg.

TABLE V.

*Effect of Phosphorus Poisoning on Blood Sugar with Administration of Galactose.*

Rabbit 2, 1.9 kilos; black male.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
1926		mg.	mg.	mg.	mg.	
Dec. 7	5.00 p.m.					2.4 mg. phosphorus in 10 cc. olive oil.
" 8	Before.	77.0	107.4	39.0		1 gm. galactose in 15 cc. water at 9.58 a.m.
" 8	10.02 a.m.		283.8	187.6		
" 8	11.05 "		126.0	60.0		
" 8	12.05 p.m.		101.8	34.4		
" 8	1.05 "		99.0	36.0		
" 8	2.51 "		123.4	33.0	132	

TABLE VI.

*Effect of Chloroform Poisoning on Blood Sugar with Administration of Galactose.*

Rabbit 19, 1.9 kilos; gray male.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
		mg.	mg.	mg.	mg.	
1926						
Dec. 12	4.00 p.m.					1.5 cc. chloroform in 10 cc. olive oil.
" 13	Before.	57.0	87.0	30.0		1 gm. galactose in 15 cc. water at 10.30 a.m.
" 13	10.32 a.m.		216.0	126.0		
" 13	11.35 "		131.2	58.0		
" 13	12.35 p.m.		110.2	31.6		
" 13	2.08 "		91.8	30.0	211	
" 14						Rabbit dead.

TABLE VII.

*Effect of Insulin on Blood Sugar with Administration of Galactose.*

Rabbit 2, 1.9 kilos; black male.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
		mg.	mg.	mg.	mg.	
1926						
Nov. 11	Before.	50.0	95.6	31.4		1 gm. galactose and 2 units of insulin in 15 cc. water at 9.45 a.m.
" 11	9.50 a.m.		242.4	150.8		
" 11	10.50 "		53.4	36.0		
" 11	11.40 "		67.4	28.2		
" 11	12.50 p.m.		111.6	30.0		
" 11	1.50		126.0	28.2	161	

the weight of the animal (1.5 to 2.2 kilos), or the volume of the solution injected (10 to 20 cc.). These considerations will prob-

ably minimize the force of possible criticisms that the dosages have not been graded according to the weight of the experimental animal. On the basis of the criterion suggested above it is to be observed that there was no striking difference between the results with 2 and 3 gm. respectively.

The results with female rabbits have not been consistent. For purposes of comparison, there were given the data for two experiments, representing the extremes of the results obtained.

In the results given in Table II, there was no apparent alteration in the rate of disposal of galactose notwithstanding the fact that none was eliminated by the kidneys. The disposal of galactose both as regards disappearance from the blood and renal excretion was presumably normal in uranium nephritis (Table III). The greatly increased urinary excretion did not produce any changes in the speed with which galactose was taken from the blood stream in phlorhizin diabetes (Table IV). Liver poisoning either by phosphorus (Table V) or by chloroform (Table VI) was accompanied by no observable alteration in the rate of disposal of circulating galactose. Insulin (Table VII) caused a striking rapidity of disappearance of galactose, as well as glucose from the circulation, without however affecting the amount of galactose lost in the urine. Except after the administration of insulin, the total sugar of the blood roughly paralleled the unfermented fraction. Control experiments for all these types of conditions except uranium nephritis are to be found in a previous communication (Corley, 1926). The difference in the figures for residual reduction is dependent on the fact that for low concentrations of reducing substances, the table of Duggan and Scott gives considerably higher values than that of Shaffer and Hartmann.

Except where otherwise stated, the urines after the termination of the experiments failed to give qualitative reduction tests, although as occasionally determined, the more delicate quantitative reactions indicated an excretion of an average of a few mg. an hour for the next 24 hours. In view of the continuous elimination of comparable amounts of reducing substances in the urine of untreated rabbits, and the difficulty of identifying galactose under these conditions, a systematic study of the urine for periods after the blood picture had returned to normal has not as yet been attempted.

In contradistinction to Bodansky's results on dogs (1923), no difficulty was encountered in preparing mucic acid from the urine following galactose administration. For example, according to the method of Fernau (1909), there was isolated from the urine of Rabbit 9 (Table I) 300 mg. of mucic acid (m.p. 205°C.).

#### DISCUSSION.

Immediately after the intravenous administration of a sugar, it is impossible to account for it all in the circulation (Cori and Goltz, 1925-26; Corley, 1926). As pointed out by Cori and Goltz, there must be a very rapid establishment of an equilibrium between the galactose in the blood and tissues. If the effect were dependent merely on an absolute capacity of the tissues, with increasing dosages the proportion of the injected carbohydrate remaining in the blood should have been markedly elevated, rather than remaining the same or even decreasing as was actually the case. While the conditions affecting the rate and state of the equilibrium established have not been determined, a number of generalizations is scarcely unwarranted. The relative rate of attaining equilibrium in the various tissues would probably be affected by the blood supply (Cori and Goltz, 1925-26), and by the rapidity of further disposal. If the latter were different than that of the organism as a whole, a compensatory shift might be expected so as to tend to maintain the equilibrium between the blood, the common intermediary, and each of the other tissues. It has been shown (Cori and Goltz, 1925-26) that the absorption of various monosaccharides in the tissues of mice was very rapid and that approximate equilibrium was attained in about a minute. The rate of disposal as evidenced by concentrations in the blood, liver, and muscles, at the end of 1, 2, and 3 minutes did not show any significant differences for the various sugars investigated. It is to be expected, however, that these differences would become more marked with longer periods of time, since certainly dependent upon rates of subsequent disposal. That galactose was not removed by the muscles so rapidly as glucose is indicated by the results of Foster (1923), who found that with the former sugar there were much smaller differences between blood sugar values in venous and finger (*i.e.* essentially arterial) blood.

Rowe (1923) has found a greater tolerance for galactose in

women than in men, and believed that the effect might be due to increased absorption by the mammary tissues. While the results obtained with female rabbits (Table I) are too inconsistent to allow any generalization, it is possible that the explanation might lie in a difference in the ease of galactose utilization at different stages of the sexual cycle or sexual development.

Presumably, renal handling of galactose is analogous to that of the other tissues, with a primary absorption, with a tendency towards equilibrium, and with excretion as the important ultimate method of disposal. Cori (1926) has found that the per cent of absorbed galactose excreted in the urine increased with increased length of absorption in spite of the fact that the rate of absorption was constant. This observation and the greater loss in urine with larger doses administered intravascularly suggest that as the tissues become more saturated, the greater amount in the circulation causes an increased urinary removal.

However, the absence of any close proportionality between the rate of disposal of galactose as evidenced by the time of practically complete removal from the blood, and the quantity metabolized, the difference between the amounts injected and excreted, suggest that galactose like glucose (Woodyatt, Sansum, and Wilder, 1915) has no absolute limit of utilization but that for every increment in the amount injected only a portion is lost in the urine. Excretion by the kidneys may probably be considered as a rather incidental method of disposal of galactose that enters the blood. This is evidenced not only by the small amount of galactose excreted normally after the intravenous injection but more strikingly so in that tartrate nephritis, involving complete retention of the sugar, had no noticeable effect on the rate at which circulating galactose disappeared.

Hamman and Hirschman (1917) have expressed the view that certain forms of nephritis may affect carbohydrate metabolism. The present observations probably may be considered to indicate that the two types of experimental nephritis employed do not exert any significant inhibitory effect on galactose utilization.

Rather surprisingly, the increased excretion of galactose (unfermented reduction) was not associated with any noticeable effect on the rate of its removal from the blood in phlorhizin diabetes. It is of interest to note that in this condition, the rate of

disappearance of galactose from the blood is quite comparable to that of the difficultly utilizable xylose (Corley, 1926). This may be considered as supporting the view suggested by Nash and Benedict (1923) that phlorhizin has an effect not only on kidney permeability but on the sugar-burning mechanism as well.

While there is considerable evidence that lactose and galactose may form extra glucose in the diabetic organism, whether the metabolic defect is due to the clinical condition, pancreas extirpation, or phlorhizin poisoning, the question as to whether galactose is completely converted to extra glucose has not been established although a number of investigators have reported that unchanged galactose was excreted, the quantities found varying from traces to 50 per cent (Brasch, 1908). The effect would undoubtedly be dependent in large measure on the completeness of the diabetes and the rate of administration of the carbohydrates. Deuel and Chambers (1925) have recently studied the rate of elimination of extra glucose in phlorhizin diabetes after the ingestion of galactose and lactose and they have reported that these compounds yield 88 and 50 per cent respectively of the theoretical amount of extra glucose. Although they do not mention testing for the unchanged carbohydrates in the urine, they certainly must have done so. While it may be merely a coincidence, attention is called to the fact that the percentage of extra sugar corresponded rather closely with the reported reducing powers of these substances (Greenwald, Samet, and Gross, 1924-25).

Although there was a marked increase in the unfermented reduction of the urine after the administration of galactose in phlorhizin diabetes, unfortunately no identification was attempted. If it can be assumed that this increase represents galactose, the greater kidney permeability might be interpreted as indicating a lowering of a renal threshold. As yet the question as to the existence of such a threshold has not been satisfactorily determined, although the balance of evidence may point to its absence. Rowe and Chandler (1924) have made the pertinent suggestion that the possibility of traces of impurities has not been ruled out as a factor causing melituria from small doses of galactose. A definite answer can probably best be obtained by determining the concentration of galactose itself in the blood that will cause the appearance in the urine of identifiable galactose. The fact that Woodyatt, Sansum,

and Wilder (1915) found that the intravenous tolerance limit for galactose was about one-ninth of that of glucose might be held as indicating the existence of a renal threshold. On the other hand, these results may merely represent the ability of the tissues to metabolize this compound without allowing enough to be lost in the urine to give qualitative reactions. However the fact that after the injection of galactose an equilibrium is established, at least for high concentrations in the blood, minimizes the force of even such a consideration.

Liver poisoning by phosphorus or chloroform had no very apparent effect on the disposal of galactose. It would be hazardous, however, to decide on this basis that the liver does not function in the metabolism of this hexose. There were lacking criteria indicating the extent of liver destruction. That this was indeed extensive in the case of chloroform poisoning, was possibly indicated by the early death of the experimental animal, although even here the contingency must not be overlooked that death resulted at least in part from toxic effects on other tissues. As a matter of fact, Williamson and Mann (1923) have shown that poisons while greatly injuring the functions of the liver did not produce complete hepatic insufficiency. They emphasize that since other tissues and organs are undoubtedly profoundly affected in many instances, and functional damage to the liver is probably not the primary cause of death, conclusions must be very carefully drawn.

Results obtained on other types of pathological and rigorous experimental alterations, must be scrutinized and evaluated no less carefully. It has been found that galactosuria usually occurred in cirrhosis of the liver and catarrhal icterus, although the association with other liver affections was less frequent (Hirose, 1912); that after Eck fistula, the nutritive value of lactose and galactose fell and it was believed that as a result of the failure of the liver to convert them to glycogen, the sugars circulated in the blood and left the body by the urine (Draudt, 1913); that the tolerance for lactose was decreased 50 per cent in chloroform poisoning (Hurwitz and Bloomfield, 1913); and that the tolerance for galactose was decreased in a number of liver disorders (Moliwa, 1914).

Bodansky (1923-24, *a*) concluded from his experiments on dogs that lowered tolerance for galactose was associated with severe



liver injury. It is to be questioned however whether the figures given warrant this interpretation. Certainly there are no striking differences between the shape or the height of the curves obtained, if the results were to be plotted. Indeed the author does state that, "owing to the low tolerance for galactose normally, any divergence from the normal as a result of liver injury would be too slight to be easily interpreted for purposes of diagnosis." The results on galactose differ markedly from those on glucose and levulose, thereby lending support to a view that the metabolism of galactose is not necessarily concurrent with that of the other two monosaccharides. On the other hand hydrazine poisoning caused an undoubted lowering of galactose tolerance (Bodansky, 1923-24, b) which may, of course, be attributable to a different action on the liver or to an effect on some other tissue or tissues.

Isaac (1920) has found that in various types of liver disorder, the rate of disappearance of fructose from the blood after ingestion was markedly slower than normal. These observations are of particular interest since they show by actual estimation of the amount of fructose circulating in the blood, that dysfunction of the liver may be reflected in the rapidity of withdrawal from the blood of a substance for the metabolism of which considerable hepatic involvement is rather generally accepted. This can but strengthen the view that the absence of any marked effect on the rate of removal of circulating galactose is to be interpreted as indicating that the liver is of no especial significance in the metabolism of this hexose. Furthermore Cori (1926) has found that although the liver absorbed galactose very rapidly from the blood, this carbohydrate played an unimportant rôle as a source of liver glycogen in the rat.

The reports of the effect of galactose on insulin intoxication have not been consistent, but this is probably explainable as due to the differences in technique, since different experimental animals and different relative doses of insulin and galactose have been employed. While the objection might be raised that *a priori* there is no adequate reason for assuming that the two phenomena are necessarily mutually reciprocal, since they probably are, in determining the interrelationship, it would seem preferable to determine the effect of insulin on galactose metabolism, as being more susceptible to quantitative measurement than the converse effect of galactose on insulin intoxication.

The results in this series of experiments are taken to indicate that the simultaneous administration of insulin increased the rate of disposal of intravenously administered galactose, yet the mechanism of this effect is not clear. Insulin may affect the rate of tissue absorption, combustion, or other method of disposal of galactose. However the failure of insulin to increase appreciably the rate of withdrawal of metabolically inactive xylose (Corley, 1926) may be considered as rendering doubtful any effect on the rate of tissue absorption unless the latter is a selective action and insulin affects such a phenomenon. Insulin may affect the galactose then either directly or by means of a coupled reaction with some substance that is susceptible to the influence of the hormone. The effects of glucose on galactose already reported are suggestive in this regard. Rather surprisingly the excretion of galactose was unaffected by the marked drop of the concentration in the blood. Any explanation of this is difficult unless possibly the galactosuria was dependent on the initial height of the galactocemia.

#### SUMMARY.

1. The rate of removal from the blood of intravenously administered galactose did not differ significantly from that of the normal rabbit in tartrate or uranium nephritis, phosphorus or chloroform poisoning, or phlorhizin diabetes, notwithstanding certain striking alterations in renal excretion.

2. The simultaneous administration of insulin was accompanied by a marked increase in the rate of removal from the blood of intravenously injected galactose in the rabbit.

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## FACTORS IN THE METABOLISM OF LACTOSE.

### II. THE EFFECT OF GLUCOSE AND GALACTOSE ON THE DISPOSAL OF INTRAVENOUSLY ADMINISTERED GALACTOSE IN THE RABBIT.\*

By RALPH C. CORLEY.

(From the Department of Bio-Chemistry of the School of Medicine, Tulane University, New Orleans.)

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The tolerance for lactose exceeds that for galactose. The usual explanation is that this is due in large measure to the influence of the glucose present in the molecule of the former. Thus it has been shown that the simultaneous ingestion of glucose decreases the excretion of galactose (Folin and Berglund, 1922). Bodansky (1923) has presented data to show that mixtures of glucose and galactose cause the appearance of a hyperglycemia in dogs considerably lower than does galactose alone. The more recent curves obtained with rabbits by Reinhold and Karr (1927) were not in agreement. Assuming, however, that glucose does exert an influence on galactose tolerance, it must be admitted that the mechanism of such an interrelationship is not clear. The possibility that the simultaneous absorption of glucose might decrease the rate at which galactose enters the blood (*cf.* Cori, 1925-26) is a factor the importance of which is regarded differently by different investigators (Benedict and Osterberg, 1923; Bodansky, 1923). There is no agreement as to whether glucose has a further and presumably more intimate influence on the metabolism of galactose. It has been considered that a profitable method of attack of this problem would be the study of the effect of glucose on galactose utilization when both substances are introduced parenterally.

Since the tolerance for glucose is increased by the previous ingestion of glucose, the question arises as to whether galactose exerts a similar influence on its own metabolism and furthermore as to whether a previous administration of glucose can affect the tolerance for galactose.

The present communication is a report of the effect on the rate of removal of galactose from the blood stream after its previous injection and after or with the intravenous administration of glucose.

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## EXPERIMENTAL.

The methods that have been employed in this investigation are those used previously (Corley, 1927). With the exception of several that had previously been poisoned with phlorhizin, the rabbits employed had been used for no other type of experiment.

TABLE I.  
*Effect of Glucose on Disposal of Galactose.*

Experiment No.....	75		22		68		13		60	
Rabbit No.....	5		5		5		17		17	
Date.....	Jan. 18, 1927		Dec. 6, 1926		Jan. 11, 1927		Nov. 22, 1926		Jan. 5, 1927	
Weight, kg.....	1.82		1.70		1.70		2.00		2.38	
Injected.										
Volume, cc.....	13		20		20		20		20	
Galactose, gm.	1		1		1		1		1	
Glucose, gm....	2		3		4		1		4	
Hours fasted.....	12		40		66		40		40	
	Total.		Total.		Total.		Total.		Total.	
	Unfermented.		Unfermented.		Unfermented.		Unfermented.		Unfermented.	
Blood sugar, mg. per 100 cc.										
Before.....	118.2	33.0	99.0	33.0	99.0	31.6	99.0	30.0	99.0	36.0
After.....	642.3	157.2	491.2	151.6	496.8	175.4	330.0	120.0	483.8	126.0
1 hr.....	141.2	44.0	208.2	63.0	288.6	47.0	114.4	47.0	115.6	44.0
2 hrs.....	100.4	30.0	101.8	30.0	140.4	28.6	91.8	30.0	101.8	36.0
3 ".....			104.6	30.0			89.4	27.0	55.0	36.0
Urine sugar, mg.	406	283	702	295	920	389	180	158	284	233

*Results.*

In order to avoid unwieldiness, only a portion of the data is presented. With a few exceptions, the simultaneous intravenous injection of glucose with galactose (Tables I to IV) was accompanied by a pronounced and unmistakable increase in the rapidity with which galactose disappeared from the blood. The glucosuria induced was associated almost invariably with a greater excretion of galactose. The circulating glucose usually returned to

normal values in 2 hours, and occasionally reached hypoglycemic levels either at that time or later. To facilitate consideration of certain general relationships, the results obtained with the same amounts of glucose have been averaged (Table V). Great divergences have been encountered in individual experiments, but a consideration of all the data does not tend to contradict the evidence of the average results.

TABLE II.  
*Effect of Glucose on Disposal of Galactose.*

Experiment No.....	43		59		68		76		23	
Rabbit No.....	6		6		6		6		6	
Date.....	Dec. 21, 1926		Jan. 5, 1927		Jan. 11, 1927		Jan. 18, 1927		Dec. 6, 1926	
Weight, kg.....	1.46		1.46		1.35		1.57		1.46	
Injected.										
Volume, cc.....	20		13		13		13		20	
Galactose, gm.	1		1		1		1		1	
Glucose, gm.....	1		2		2		2		3	
Hours fasted.....	40		40		66		12		40	
	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.
Blood sugar, mg. per 100 cc.										
Before.....	91.8	30.0	104.6	30.0	27.0	84.4	30.0	84.4	33.0	
After.....	427.6	170.2	440.6	205.6	482.4	205.6	504.2	163.8	504.6	151.6
1 hr.....	123.4	34.6	203.0	47.0	213.4	44.0	162.4	37.6	303.0	63.0
2 hrs.....	84.4	27.0	104.6	30.0	174.2	30.0	104.6	30.0	182.8	39.0
3 ".....	108.8	27.0	84.4	30.0					115.6	33.0
Urine sugar, mg.	333	228	291	150	714	310	458	250	795	450

Experiment 71 (Table IV) typifies the results obtained with animals that had previously been poisoned with phlorhizin, but had recovered at least so far as the absence of glucosuria may be taken as evidence. Under these conditions, there was no indication that the greater urinary excretion caused an increased rate of removal of galactose from the blood. On the other hand, in the case of a rabbit that had been fed oats *ad libitum* an hour previously

(Experiment 7, Table III), while there was no excretion of fermentable carbohydrates, there was a hyperglycemia and an apparently more rapid disposal of galactose in the blood. There was no increased rate of disappearance of circulating galactose, when injected either during (Table VI) or directly after the duration of the hyperglycemia resulting from the intravascular introduction of glucose.

TABLE III.  
*Effect of Glucose on Disposal of Galactose.*

Experiment No.....	52		65		82		49		7	
Rabbit No.....	31		31		31		32		5	
Date.....	Jan. 3, 1927		Jan. 11, 1927		Jan. 18, 1927		Dec. 23, 1926		Nov. 15, 1926	
Weight, kg.....	1.70		1.58		1.93		2.04		1.70	
Injected.										
Volume, cc....	18		18		18		13		10	
Galactose, gm.	1		1		1		1		1	
Glucose, gm....	3		3		3		0.5			
Hours fasted....	24		66		12		60		0	
	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.
Blood sugar, mg. per 100 cc.										
Before.....	89.4	27.0	95.4	27.0	89.4	27.0	83.2	30.0	178.0	33.0
After.....	483.8	159.4	403.8	143.0	569.0	120.8	318.6	133.8	307.8	170.2
1 hr.....	107.4	36.0	216.0	49.0	131.2	33.0	149.4	44.0	144.2	43.0
2 hrs.....	63.0	24.0		25.6	96.6	27.0	120.8	27.0	120.8	28.6
3 ".....							115.6	27.0	143	27.0
Urine sugar, mg..	522	250	508	260	718	285	135	132	150	150

The previous administration of galactose was not effective in increasing the rapidity of disposal of a subsequent dose (Tables VII to IX). The residual reduction at the end of an hour, Table IX, was the lowest that has been observed in a considerable number of experiments of this type.

## DISCUSSION.

While in the large majority of cases the simultaneous administration of glucose has been found to increase the rate of disposal of circulating galactose, the data are not adequate to furnish an entirely satisfactory explanation of this effect. Although the objection is unavoidable that the individual differences in the con-

TABLE IV.  
*Effect of Glucose on Disposal of Galactose.*

Experiment No.....	50		69		75		71	
Rabbit No.....	15		15		15		22*	
Date.....	Jan. 3, 1927		Jan. 11, 1927		Jan. 18, 1927		Jan. 11, 1927	
Weight, <i>kg.</i> .....	1.82		1.70		1.70		1.80	
Injected.								
Volume, <i>cc.</i> .....	13		20		22		13	
Galactose, <i>gm.</i> .....	1		1		1		1	
Glucose, <i>gm.</i> .....	1		4		5		2	
Hours fasted.....	12		66		12		15	
	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.
Blood sugar, <i>mg. per</i> <i>100 cc.</i>								
Before.....	94.2	31.6	103.2	31.6	120.8	27.0	101.8	27.0
After.....	400.0	178.0	503.2	139.0	780.0	113.0	460.2	167.6
1 hr.....	152.0	60.0	315.0	36.0	251.4	44.0	158.6	58.0
2 hrs.....	91.8	33.0	152.0	31.6	139.0	27.0	101.8	28.6
3 ".....	110.2	28.6						
Urine sugar, <i>mg.</i> ....	267	220	838	378	907.2	390	406	213

\* Rabbit 22 previously poisoned with phlorhizin, Dec. 13, 1926.

ditions of the experiments and the results obtained, tend to vitiate the value of the evidence of the average results, there must be significance in the rather consistent variations of the latter with different dosages of glucose (Table V). The importance of the increased excretion of galactose associated with the induced glucosuria must not be minimized. Indeed, from a consideration of the



data obtained, there would seem to be no logical reason for doubting that the administration of large quantities of glucose may cause a more rapid disappearance of galactose from the blood by virtue of the increased loss through the kidneys.

It is not surprising that a renal loss of about 500 mg. of galactose (390 mg. as glucose) in Experiment 75 (Table IV) should be so effective in decreasing the concentration in the blood, and yet an even greater excretion in Experiment 23 (Table II) caused

TABLE V.  
*Effect of Glucose on Disposal of Galactose. Average Results.*

No. of experiments...	7		8		8		6		3	
Weight, kg.....	1.78		1.68		1.93		2.00		1.98	
Injected.										
Volume, cc....	12.8		15.8		18.0		20.0		22.0	
Galactose, gm..	1		1		1		1		1	
Glucose, gm....	1		2		3		4		5	
Hours fasted....	45		30		38		40		30	
	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.	Total.	Unfermented.
Blood sugar, mg. per 100 cc.										
Before.....	95	30	98	30	98	28	102	31	104	29
After.....	386	157	500	161	480	139	501	138	669	114
1 hr.....	128	49	163	41	198	41	237	44	228	38
2 hrs.....	96	30	110	30	116	28	130	33	123	31
3 " .....										
Urine sugar, mg.	230	182	466	258	591	263	721	299	897	404

no similar effect on the blood level. This latter fact that increased elimination of galactose by the kidney may not be accompanied by a greater lowering of the blood value (Experiments 22, 23, 50, and 71) and the further observations that with Rabbit 31 (Experiments 52, 65, and 82) markedly different blood values may be associated with similar renal loss of galactose, probably indicate that other factors than the renal effect of the glucose were operative. In this regard one may consider an alteration of the

TABLE VI.

*Effect of Previous Administration of Glucose on Disposal of Galactose.*

Rabbit 13, 1.82 kilos, white male, fasted 40 hours.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
<i>1928</i>		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
Dec. 6	10.50 a.m.	58.1	151.6	28.4		3 gm. glucose in 20 cc. water injected at 10.58 a.m.
" 6	11.03 "		483.8	25.6		
" 6	11.51 "		329.4	28.6		649 mg. total reduction. 1 gm. galactose in 15 cc. water injected at 12.53 p.m.
" 6	12.48 p.m.		185.2	30.0	95.0	
" 6	12.55 "		339.0	182.8		
" 6	1.50 "		185.2	79.2		
" 6	2.50 "		144.2	42.0		164 mg. total reduction.
" 6	3.50 "		133.8	27.0	153.0	

TABLE VII.

*Effect of Previous Administration of Galactose on Disposal of Galactose.*

Rabbit 15, 1.70 kilos; brown male, fasted 36 hours.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
<i>1928</i>		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
Nov. 17	9.45 a.m.	52.9	110.2	30.0		1 gm. galactose in 15 cc. water injected at 9.50 a.m.
" 17	9.55 "		275.2	157.2		
" 17	10.55 "		161.2	71.0		1 gm. galactose in 15 cc. water injected at 11.57 a.m.
" 17	11.55 "		128.6	33.0	139	
" 17	12.00 m.		293.4	179.2		
" 17	1.00 p.m.		171.6	80.6		
" 17	2.00 "		153.4	40.4		
" 17	3.00 "		137.0	27.0	149	
" 18	3.00 "				123	

TABLE VIII.

*Effect of Previous Administration of Galactose on Disposal of Galactose.*

Rabbit 21, 2.0 kilos; gray male, fasted 60 hours.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
1926		mg.	mg.	mg.	mg.	
Nov. 30	10.55 a.m.	48.7	89.4	30.0		2 gm. galactose in 20 cc. water injected at 11.05 a.m.
" 30	11.08 "		336.6	263.6		
" 30	11.50 "		208.2	131.2		
" 30	1.00 p.m.		139.0	69.0		1 gm. galactose in 15 cc. water injected at 2.10 p.m.
" 30	2.07 "		101.8	33.0	370	
" 30	2.12 "		283.8	172.8		
" 30	3.12 "		152.0	76.6		
" 30	4.10 "		118.0	33.0		
" 30	5.10 "		139.0	24.0	110	
Dec. 1	5.00 "				88	

TABLE IX.

*Effect of Previous Administration of Galactose on Disposal of Galactose.*

Rabbit 9, 2.15 kilos; gray male, fasted 40 hours.

Date.	Time.	Blood per 100 cc.			Urine. Unfermented reduction.	Remarks.
		Non-protein nitrogen.	Total reduction.	Unfermented reduction.		
1926		mg.	mg.	mg.	mg.	
Dec. 6	11.00 a.m.	50.3	96.6	30.0		3 gm. galactose in 22 cc. water injected at 11.08 a.m.
" 6	11.13 "		438.0	351.0		
" 6	11.54 "		271.2	180.4		
" 6	1.13 p.m.		165.0	79.2		1 gm. galactose in 15 cc. water injected at 2.12 p.m.
" 6	2.10 "		128.6	30.0	826	
" 6	2.14 "		293.4	172.8		
" 6	3.14 "		159.8	52.0		
" 6	4.14 "		152.0	30.0		
" 6	5.14 "		144.2	28.4	209	
" 7	5.00 "				110	

ability to handle galactose by itself or in conjunction with glucose. However, in this series of studies there have been found no marked variations in the ability to handle galactose in fasting rabbits of approximately the same size or in the same rabbits at different times. While the influence of a renal threshold varying in different animals and in the same animal at different times would furnish a satisfactory explanation of the lack of a consistent relationship between blood values and renal excretion, the doubt even as to the existence of a renal threshold must leave this consideration in abeyance at present.

Experiments could be chosen from this group to be cited as proof that glucose increased, decreased, or had no effect on galactose metabolism. Thus increased renal excretion with blood figures comparable to those obtained with galactose alone may well be considered as evidence that glucose inhibits the metabolism of galactose. It is indeed conceivable that overwhelming the organism with glucose might decrease the ease of handling another sugar, even though smaller amounts might actually have the opposite effect. While presumably there is no limit except death, to the amount of glucose or galactose that the tissues can absorb and metabolize, there have been presented no data as to whether this same generalization holds for each of a mixture of carbohydrates. In this connection, it is possible that otherwise discrepant results might be attributed to differences in what constitutes an overwhelming dose. The nutritive condition would certainly be a factor in this regard.

Attention is called to the fact that the average of the results obtained with 1 gm. of glucose were quite similar to those with galactose alone (Corley, 1927). The residual reduction at the end of an hour came within the lower limit of the range previously observed, though the galactose in the urine was greater. On the other hand individual experiments with 1 gm. and one with 0.5 gm. of glucose (Experiment 49, Table III) showed the galactose in the blood at the end of an hour rather low. The findings with several rabbits that had previously been poisoned with phlorhizin and presumably later recovered were interesting and suggest that there existed some impairment of galactose metabolism. It is felt that the significance of these results is enhanced by the fact that *all* the results with the phlorhizinized rabbits were consistent, none of the

blood curves for galactose being lower than those for galactose alone in the normal animal notwithstanding the greater excretion. In contradistinction to this, in the normal animal it is to be noted that the high residual reductions at the end of an hour occurred rarely and never in *all* the experiments with the same rabbit. These results resemble those obtained in phlorhizin glucosuria (Corley, 1926). To determine whether both types of experiments are to be taken as indicating a permanent impairment (or at least one lasting beyond the time of disappearance of the glucosuria) of the metabolism of galactose alone or as interrelated with that of glucose, further experimentation is necessary. Unfortunately no studies have been carried out on the rate of disposal of galactose alone, after seeming recovery from phlorhizin diabetes.

The rate of disposal of circulating galactose was not increased, following an injection of glucose, although the glucose of the blood was still at hyperglycemic levels (Table VI). This supports the interpretations either that parenterally introduced glucose does not affect the metabolism of galactose through a simultaneous interreaction or as a consequence of some stimulation, or that there is merely an inadequate or delayed stimulation.

The previous administration has not been found to cause any increased rate of disappearance of galactose from the blood. In fact, the results could better be interpreted as indicating that there occurs a slight slowing of the rate of removal, the effect if existent probably to be attributed not to any actual inhibition but to the possibility that certain amounts of galactose remaining in the tissues would increase the actual size of the dose to be handled.

After the previous disposal of galactose, the excretion following a subsequent dose was little affected in most cases. A slight increase might be attributable to the factor mentioned above, unmetabolized galactose remaining in the tissues. The same explanation might be applied to the findings of Cori (1926) that the per cent of absorbed galactose excreted in the urine increased with increasing length of absorption.

Taking into account the small excretion of unfermented reducing substances in the urine for the next 24 hours, and the presence of such substances almost invariably in rabbit urine, there is considerable justification for the belief that very little galactose

escapes utilization after the residual reduction of the blood returns to normal.

Reinhold and Karr (1927) have decided on the basis of curves obtained with rabbits, that a previous ingestion of galactose decreased the height of the hyperglycemia resulting from the subsequent administration of either glucose or galactose. Disregarding the objection that the results are for total reduction in the blood, the effect is quite pronounced with regard to glucose, but extremely doubtful in the case of the galactose. Second increases of 84 and 82 mg. per 100 cc. following initial rises of 96 and 66 mg. respectively scarcely justify the statement that, "the rise following the second sugar [was] perceptibly less than the initial increase." The figures quoted are necessarily approximations taken from the graphs presented, but it is believed that they do no essential injustice.

The tolerance for galactose has been reported to decrease with increased length of fasting (Reinhold and Karr, 1927). In the present study, it was observed that the rabbits subjected to but short periods of fasting, 12 hours, seemed in general to make a more rapid disposal of galactose, but the relationship did not hold for those fasted a longer period of time (*cf.* Rabbit 31, Table III). A partial explanation of this latter consideration may possibly lie in the fact that in the absence of a standardization of the previous diet, the time of fasting is a poor criterion of the animal's nutritive condition, which latter might be expected to be the significant factor. This general effect, however, might be due to a decreased store of glucose, decreased formation or mobilization of insulin, or a decreased ability of the organism to metabolize galactose due to some other relationship.

It was rather surprising to find a lack of correspondence between the rates of disposal of galactose when injected during hyperglycemias resulting from food in the digestive tract and from glucose injected during a fast. This may be attributable to several possibilities, evaluation of which is handicapped by insufficient data. It may signify merely a relative or an absolute difference in the factors involved.

Thus it is conceivable that the ability of glucose to stimulate the further metabolism of itself and possibly that of other carbohydrates is dependent on some property that distinguishes ab-

sorbed from parenterally introduced glucose. There can be little question, however, of the ability of intravenously administered glucose to exert a stimulating influence on the metabolism of a successive dose (du Vigneaud and Karr, 1925; Jordan, 1927).

The increased excretion of galactose accompanying that of glucose when both hexoses are injected is not readily explained. It is conceivable that the passage of large amounts of glucose might injure the renal cells so as to increase the permeability for galactose, or, what possibly is not essentially very different, that one carbohydrate might affect the threshold for another. Evidence opposing this latter conception may be found in the fact that following the intravenous injections of large amounts of galactose, there has been found no appreciable excretion of glucose (*i.e.* fermenting sugar) notwithstanding the hyperglycemic levels of the total blood sugar. It is to be admitted however that these conclusions might be altered by a repetition of these experiments with more delicate methods of urine analysis.

While the introduction of large quantities of glucose may increase the rapidity with which galactose disappears from the blood by causing a greater renal elimination, this factor obviously cannot account for the effect of glucose on galactose tolerance, reports of which have been based on decreased melituria.

#### SUMMARY.

1. No evidence has been found that glucose administered intravenously increases the ability of the rabbit to metabolize circulating galactose. The increased rate of disappearance of galactose from the blood can be explained at least in large measure by the greater urinary excretion.

2. A previous intravenous administration of glucose has not been found to increase the ability of the rabbit to dispose of circulating galactose.

3. A previous intravenous administration of galactose has not been found to increase the ability of the rabbit to dispose of a subsequent dose.

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# THE FATE OF CERTAIN HETEROCYCLIC RING COMPOUNDS IN THE ANIMAL BODY.

By N. JEAN NOVELLO.

*(From the Chemical Laboratories of Hunter College, New York.)*

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The object of this experiment was to make a quantitative study of the methylation of certain organic compounds in the animal body, and to compare it with the numerous examples of methylation which ordinarily take place in the plant kingdom. Methylation is rather unusual in the animal body, so that the experiment deals mainly with the fate of certain heterocyclic compounds in the animal body. The work of His on acetylation was repeated with varying degrees of success (1). The work of Ackermann seemed to be better suited for this method of experimentation and in fact was much more satisfactory from every view-point (2). The compounds picked for study were pyridine, pyrrole, pyrrolidine, and imidazole. The animals used as subjects were dogs and rabbits. It was hoped that the work might be extended to humans but the compounds were found much too toxic for this.

## EXPERIMENTAL.

It is commonly supposed that rabbits do not possess the power of adding on a methyl group to compounds ingested. Therefore, it was necessary to try the action of pyridine on the metabolism of the rabbit. Two sets of experiments were conducted, one in which the course of nitrogen and sulfur metabolism was studied after varying quantities of pyridine had been injected, and a second experiment in which attempts were made to isolate a methylation product from the urine after the maximum dose had been fed. Pyridine was injected in doses of 0.25, 0.5, 1, and 1 gm. on alternate days over a period of 8 days. The urine was collected in 48 hour periods. Figures for both the sulfur and nitrogen show that a dose of 0.5 gm. of pyridine has no appreciable effect on either the

## 34 Metabolism of Heterocyclic Compounds

endogenous or exogenous metabolism of the rabbit. After an increase of the dosage to 1 gm. there is a decided increase in the endogenous catabolism of the rabbit. There is no reason to believe that any appreciable amount of pyridine is excreted as ethereal sulfate since there is less than 100 per cent increase in this form of sulfur after the heaviest dose, and this would only account for less than 1 per cent detoxication of the total amount. Attempts to isolate a methylated product from the urine met with failure after employing both the methods of His and Ackermann. Since methylation does not take place in rabbits (but does in dogs), while acetylation does take place in rabbits (but does not in dogs), it seemed that perhaps the pyridine might have been acetylated in the body of the rabbit. Samples of the rabbit urine were boiled with sulfuric acid in order to hydrolyze any acetyl derivative which might be present, but no acetic acid was obtained. The pyridine under these conditions would remain in the flask as an additive sulfate, thereby not masking any acetic acid liberated. The latter should be detected by its vinegar-like odor. The physical chemical properties of pyridine acetate make it practically impossible otherwise to detect the small quantities of this substance which would have been present. It was also found that the greater part of the pyridine is rapidly eliminated—the greater part of this being eliminated via the skin, lungs, and feces, while a small percentage only is eliminated in the urine and this as free pyridine.

### *Imidazole.*

This heterocyclic compound, an important constituent of the essential amino acid, histidine, has recently been shown by Sherwin and coworker (3), also by Rose and Cox (4), to be inadequate as a substitute for histidine itself. It is impossible, however, to study the metabolism of such a compound since it is known to have considerable pharmacological importance and is believed by many to be possible of synthesis in the living cell through a condensation of ammonia with some carbohydrate substance, perhaps glyoxal, and seems to be easily synthesized *in vitro* through the action of formaldehyde and ammonia. Imidazole of Kahlbaum manufacture was chosen for this work. Total nitrogen, ammonia nitrogen, urea, and uric acid were determined

in the urine. Total sulfur, ethereal sulfur, and reduced sulfur were also determined. The possibility that through oxidation a hydroxy compound might be formed was taken into consideration. In this latter case, it would have required enormous amounts of sulfate or glycuronic acid for its detoxication. Results showed that considerable amounts of this substance are excreted unchanged in the urine. There is no doubt but a small amount is excreted as uric acid. It is questionable whether any is excreted as ammonia. A considerable increase in the ammonia output after feeding imidazole in even 0.5 gm. doses may be due to the marked increase in general catabolism. The substance is extremely soluble, but still more soluble when converted into the hydrochloride or sulfate. It is quickly absorbed and as quickly eliminated. The ethereal sulfate figures and lack of reduction in the urine are conclusive evidence of the lack of formation of a heterocyclic alcohol. Apparently considerable of the nitrogen resulting from the destruction of the ring is converted into urea. This brings up the old contention regarding the precursors of uric acid and urea. There appears here some support for Hopkins' assertion (5) that histidine and arginine form as much or more uric acid than they do urea. If this be the case, it is most likely that the nitrogen of the  $\alpha$ -amino group, like that of all the other  $\alpha$ -amino acids, is converted into urea. The nitrogen of the remainder of the molecule is very probably to a large extent converted into uric acid. Various attempts were made to determine the presence of a possible methyl imidazole. Each attempt was met with failure.

#### *Pyrrole.*

This compound was found to be very toxic. It has the power of forming additive salts with all mineral acids. It was injected subcutaneously in 0.5 gm. doses as the pyrrole hydrochloride. Rabbits were injected on alternate days and died the day following the injection of the third dose. Attempts were made to detect methyl or acetyl derivatives but without success. In each case it was found that a great deal of the compound was destroyed. The compound was quickly eliminated either as pyrrole or as some other catabolic end-product. Uric acid figures show that practically none of the nitrogen goes to form this end-product. The

same holds for ammonia nitrogen, for which figures are negative throughout. A comparison of the urea figures and the total nitrogen figures shows that 40 to 50 per cent of the pyrrole nitrogen goes over in the form of urea. While there is no proof, save that of the process of elimination, it seems that the nitrogen not accounted for as urea nitrogen is no doubt excreted in the form of unchanged pyrrole. Apparently there is no oxidation in the ring with the formation of a secondary or tertiary alcohol (pyrroxyl), because if such had been the case, there would no doubt have been a decided increase in the ethereal sulfate output or a decided reducing power of the urine, resulting from the detoxication of such a compound by means of either sulfuric or glycuronic acids respectively.

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## DIETARY REQUIREMENTS FOR REPRODUCTION.

### VIII. FURTHER STUDIES OF A SKIMMED MILK POWDER REPRODUCTION-DEFICIENT DIET.\*

By BARNETT SURE.

*(From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville.)*

(Received for publication, March 23, 1927.)

Since 1906 considerable progress has been made in demonstrating the existence of hitherto unrecognized dietary components that exert stimulating effects on growth, and the deficiency of which produces certain dietary diseases, such as beriberi, scurvy, and rickets. Relatively less progress has, however, been made in studies of the dietary requirements for fertility and lactation. The difficulties encountered are many, and a conflicting literature is already developing because of different methods of biological technique adopted by various investigators.

In previous communications (1) extensive evidence has been presented showing the beneficial effects on fertility and lactation obtained by additions of wheat oil, or very small amounts of unsaponifiable matter therefrom to a skimmed milk powder reproduction-deficient diet. Recently Anderegg and Nelson (2) claim to have disproved the vitamin E theory based on studies with skimmed milk powder diets. They state that the difficulties which I encountered in reproduction are entirely due to faulty dietary management, that the cod liver oil in the ration reacting with the skimmed milk undergoes decomposition, and that there is a toxicity produced due to the formation of acrolein. By the simple method of adding 5 per cent of distilled water in the ration, removing cod liver oil and the Harris yeast from the diet, and administering the latter two components of the diet separately, they have secured fourth generation young.

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## 38 Dietary Requirements for Reproduction. VIII

My experiences on the effect of the removal of cod liver oil from the basal skimmed milk powder sterility diet on fertility and lactation have already been reported (1). While such a procedure is at times, but not regularly, followed by some of the females carrying their young to term, the failure in lactation is 100 per cent. We have now completed lactation studies of several hundred litters, employing the same technique, and not one litter was successfully weaned during the nursing period. We have spared no efforts in this laboratory to determine, if possible, the

TABLE I.  
*Harris Yeast as Source of Vitamin E.*

Ration No.	Yeast.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Remarks.
	<i>per cent</i>	<i>days</i>	<i>days</i>			
841	1.0	131	79	3	0	Basal skimmed milk powder ration with cod liver oil removed. 0.3 cc. cod liver oil administered daily to each animal.
842	1.0	120	72	3	0	Same basal ration and dosage of cod liver oil as in Ration 841. At breeding increased Harris yeast from 1.0 to 2.0 per cent in ration.
843	2.0	162	82	3	0	Same basal ration and allowance of cod liver oil as in Ration 841.
844	3.0	116	61	2	0	" "

cause of this discrepancy. Since Anderegg and Nelson (2) have employed higher concentrations of Harris yeast, we have performed experiments in which Harris yeast was fed in 3 to 5 times the proportion employed in previous work as a source of vitamin E. Table I shows that out of 11 females that received cod liver oil separately from the ration and large proportions of Harris yeast none produced young.

The following are results of experiments planned to duplicate the technique of Anderegg and Nelson as closely as possible.

*Ration 842.*—After three females failed to become pregnant

during a reproduction period of 65 days on our basal diet containing 1.0 per cent Harris yeast, 0.3 cc. of cod liver oil being administered separately from the ration daily to each animal, the diet was changed, so that 5 per cent distilled water replaced an equivalent amount of dextrin. The yeast was removed from the diet. In order to circumvent the possibility of encountering at this time male sterility, the females were rebred with two stock males. False bottom screens were removed. The yeast and cod liver oil were triturated with a little distilled water and administered in Petri dishes daily. 29 days after this change in the diet was made no signs of advanced pregnancy were apparent. During the second reproduction period, 1400 gm. of ration and 30 gm. of cod liver oil and 30 gm. of Harris yeast were consumed. The animals became filthy and it was difficult to keep shavings from being scattered in Petri dishes containing vitamin. The experiment was, therefore, discontinued sooner than planned.

*Ration 843.*—Three females in this experiment (see Table I) were bred July 24, 1926. No signs of advanced pregnancy were observed on September 24, at which time the ration was changed, so that 5 per cent distilled water replaced an equivalent amount of dextrin, and the Harris yeast and cod liver oil were triturated with a little distilled water and administered separately from the ration in Petri dishes. The animals were allowed to stay on the false bottom screens. The two males were replaced by stock males. No signs of advanced pregnancy were observed as late as November 4, neither did any of the females at any time during the entire reproduction period show a resorption curve (3). During the second reproduction period 2000 gm. of basal ration were consumed, and 40 gm. of cod liver oil and 40 gm. of Harris yeast.

Employing a refined cod liver oil product, secured from Seapure Products Company, Portland, Maine, I have been unable to confirm the findings of Anderegg and Nelson.

The vitamin E theory questioned in 1924 by Hogan and Harshaw (4) has recently been accepted by these investigators (5). Profiting by my results reported on the vitamin E content of cottonseed oil, they have removed Crisco (which is a hydrogenated cottonseed oil product) from their basal rations, and have since obtained entirely different experimental results on reproduction.



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TABLE II.

*Effect of Additions of Wheat Oil, Heated at Various Temperatures in Presence and Absence of Current of Air, to Basal Skimmed Milk Powder Reproduction-Deficient Diet on Fertility and Lactation.*

In Rations 557 and 556 the cod liver oil was left in ration; in all other rations the cod liver oil was removed from ration and 0.3 cc. of that oil given daily to each animal.

Ration No.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Wheat oil.	Treatment of wheat oil and remarks.
	days	days						per cent	per cent		
557	104	52	3	0	0	0	0	0	0	1.0	Aerated 24 hrs. at 100°C. Excellent growth; all females sterile.
556	110	41	3	0	0	0	0	0	0	3.0	Aerated 24 hrs. at 100°C. One female died after 15 days on experiment. Two females made very good growth but neither showed any signs of advanced pregnancy the entire reproduction period.
827	110	97	3	3	23	23	18	18	100	3.0	Heated 24 hrs. at 37°C.
828	145	100	3	3	31	31	18	11	61	3.0	Aerated 24 hrs. at 37°C. One female disposed of its litter few days after delivery.
830	133	99	3	3	31	31	18	17	94	3.0	Heated 24 hrs. at 50°C.
829	97	64	3	0	0	0	0	0	0	3.0	Aerated 24 hrs. at 50°C.
831	85	51	3	3	23	23	18	11	61	3.0	Heated 24 hrs. at 75°C. One female devoured its young 2nd day after birth.

TABLE II—*Concluded.*

Ration No.	Duration of experiment.		Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Wheat oil.	Treatment of wheat oil and remarks.
	days	days								per cent	per cent	
832	84	34	3	0	0	0	0	0	0	0	3.0	Aerated 24 hrs. at 75°C. One male and two females died 3 wks. after experiment was begun.
833	99	69	3	2	11	11	8	0	0	0	3.0	Heated 24 hrs. at 100°C. One male died after being on experiment 3 wks. One female died after being on ration 50 days. Weight 178 gm. when found dead in cage, having gained 102 gm. during 50 days of experiment.
834	83	50	3	0	0	0	0	0	0	0	3.0	Aerated 24 hrs. at 100°C. Subnormal growth from time of mating.

Table II shows the results of further studies on the thermostability of vitamin E. It is perfectly clear that addition of wheat oil aerated at temperatures above 50°C. has a deleterious effect on lactation, but since we have recently also found such oxidized oil to result fatally during the growing period, it is possible that the injurious effect of the oxidized oil fed at a 3 per cent level may be brought about by peroxides of the wheat oil toxic to the organism. At least a differentiation of a galactagogic vitamin associated with vitamin E, based on such experimental findings, cannot be made. Much more convincing evidence is needed.

Since the preparation of large amounts of wheat oil and unsaponifiable matter therefrom is a laborious and expensive process,

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we next turned our efforts towards the possible finding of a cheaper source for vitamin E studies. A preparation of unsaponifiable matter from crude cottonseed oil was biologically examined by a method previously outlined (1).

### *Preparation of Unsaponifiable Matter from Crude Cottonseed Oil.*<sup>1</sup>

2 gallons of crude cottonseed oil were mixed with 8 liters of alcoholic KOH containing 5 pounds of KOH. The mixture was allowed to stand in the room 2 days. The soap was then diluted by adding 15 gallons of ether and 8 gallons of distilled water. 5

TABLE III.

*Unsaponifiable Matter from Crude Cottonseed Oil as Source of Vitamin E.*

1 gm. of concentrate equals 170 gm. of oil. Dextrin of rations carried the cottonseed oil concentrate.

Ration No.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Amount of unsaponifiable matter in ration.
	days	days							per cent	per cent
716	104	56	3	3	10	5	5	0	0	0.01
715	81	32	3	2	14	11	10	0	0	0.01
713	102	36	6	1	4	2	2	0	0	0.03
714										
711	95	36	6	4	10	8	8	0	0	0.05
712										
710	115	56	3	3	25	19	19	3	16	0.10

gallons of ether were decanted, 5 gallons more of ether added, then decanted eight times. The ether was evaporated to 1 gallon, then transferred to a vacuum. The residue thus obtained was dissolved in ethyl alcohol, 1500 cc. containing 100 gm. of KOH, and held at 37.5°C. for 4 hours. It was then diluted with water and the unsaponifiable matter again shaken out with ether. The ether was evaporated and the crystals insoluble in ethyl alcohol separated. The crystals were dissolved in 500 cc. of boiling alcohol, cooled, and the filtrate added to the original, which was then

<sup>1</sup> The unsaponifiable matter was prepared by Mr. E. H. Stuart, chemist of the Research Laboratories of Eli Lilly and Company, Indianapolis.

TABLE IV.

*Unaponifiable Matter from Orude Cottonseed Oil as Source of Vitamin E.*

1 gm. of concentrate equals 170 gm. of oil. Cod liver oil carried the cottonseed oil concentrate.

Ration No.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	days	days							per cent	
819	153	109	3	3	27	27	18	6	33	Cod liver oil removed from ration; 0.3 cc. of that oil, containing 10 mg. cottonseed oil concentrate, per animal per day.
820	164	120	3	2	13	13	12	5	42	Cod liver oil left in ration. Unaponifiable matter from cottonseed oil dissolved in cod liver oil, so that each cc. of latter oil contained 10 mg. of concentrate. Approximately 0.07 per cent concentrate in ration.
821	92	48	3	2	13	8	5	0	0	Cod liver oil removed from ration; 0.3 cc., containing 5 mg. cottonseed oil concentrate, per animal per day.
822	163	115	3	3	23	23	18	6	33	Cod liver oil left in ration carried 0.035 per cent cottonseed oil concentrate (as percentage of total ration). Approximately 5 mg. concentrate per animal per day.
823	73	26	3	2	8	5	5	0	0	Cod liver oil removed from ration; 0.3 cc., containing 2.5 mg. cottonseed oil concentrate, per animal per day.
824	106	58	3	3	22	13	10	0	0	Cod liver oil left in ration carried 0.0175 per cent cottonseed oil concentrate, or approximately 2.5 mg. concentrate per animal per day.

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evaporated *in vacuo* to dryness. 1 gm. of concentrate is equal to 170 gm. of cottonseed oil.

Tables III and IV summarize the biological results secured on fertility and lactation. It is clear from these tables that cod liver oil is a more efficient carrier of the unsaponifiable matter from crude cottonseed oil as a source of vitamin E than is dextrin. This is evident from the total number of young born and the per cent of young weaned. Table IV shows that as little as 0.0175 per cent of unsaponifiable matter from crude cottonseed oil in the basal skimmed milk powder-cod liver oil-containing diet, on which we have never obtained a litter of young, is sufficient to overcome the resorption of the fetus during gestation. It is hardly possible to believe that the very small amounts of unsaponifiable matter contained in Ration 824 (Table IV) could have acted in the form of an antiseptic preventing the decomposition of cod liver oil in the basal ration.

### SUMMARY.

Evidence is presented showing that very small amounts of unsaponifiable matter from crude cottonseed oil (0.0175 per cent in the ration) added to a skimmed milk powder reproduction-deficient diet, prevent female sterility; also that larger amounts (0.035 per cent in the ration) of such unsaponifiable matter added to the same diet are followed by beneficial effects on lactation.

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## DIETARY REQUIREMENTS FOR REPRODUCTION.

### IX. COD LIVER OIL VERSUS WHEAT OIL AS SOURCES OF VITAMIN E.\*

By BARNETT SURE.

WITH THE TECHNICAL ASSISTANCE OF H. M. BOGGS.

*(From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville.)*

(Received for publication, March 23, 1927.)

The fundamental argument on which the vitamin E theory was postulated by the author (1) was based on the fact that experimental animals receiving diets containing liberal amounts of fat-soluble vitamins A and D were experiencing considerable difficulty in reproduction. That cod liver oil is deficient in the reproductive factor has been reported by Evans and Bishop (2), Sure (3), Mattill (4), Hartwell (5), and Hogan and Harshaw (6). Recently Nelson and Jones (7), in a preliminary report, have made the statement: "If butter fat contains vitamin E, then cod liver oil is a much richer source of this vitamin."

Since cod liver oil from various sources, possibly refined by different methods of manufacture, may vary considerably in potency of fat-soluble vitamins, it is necessary at this time to point out the exact source of the cod liver oil employed in vitamin investigations in this laboratory. In 1921 we began employing a refined cod liver oil, supplied by Lord Brothers, Portland, Maine.<sup>1</sup> The manufacturers, now known under the name of Seapure Products Company, in a recent communication, have informed us that the oil is expressed from fresh, hard livers, at a temperature of 130°F. after the livers have received a good washing and the waste is removed. No change in the process of manufacture since 1921 is mentioned in their communication.

\* Research paper No. 45, Journal Series, University of Arkansas.

<sup>1</sup> Recommended by Dr. Steenbock of the University of Wisconsin.

That the cod liver oil employed in our vitamin studies is very abundant in fat-soluble vitamins A and D, we have determined with considerable precision on growth experiments. The difficulty of exhausting our young of fat-soluble vitamins from weaning age, due to storage from mothers receiving a stock diet containing 1 per cent of cod liver oil, has also served as another index of evaluating the potency of our product.

TABLE I.  
*Composition of Rations.*

Ration No.	Skimmed milk powder.	Purified casein.	Commercial casein.	Agar-agar.	Salts 32.	Ferric citrate.	Wheat oil.	Harris yeast.	Defatted wheat embryo.	Dextrin.
835	50.0			2.0		0.2	3.0	1.0*		43.8
836										
837										
838	50.0			2.0		0.2	0.0	1.0*		46.8
839										
840										
956	20.0			2.0	4.0		0.0	5.0		69.0
957										
958										
959										
867			20.0		4.0	0.25	0.03		30.0	45.72
868			20.0		4.0	0.25	3.03		30.0	42.72

\* From weaning time up to the beginning of the reproduction period the rations contained 0.4 per cent Harris yeast. The increase was made at the time of mating and that percentage left in the rations until the termination of the experiments.

The experiments reported in this paper on cod liver oil as the only source of vitamin E have extended over a period of more than 2 years, the results of which are summarized in Tables I to V. The biological assay was conducted with three types of diets: (1) The basal skimmed milk powder reproduction diet, from which the cod liver oil was removed and the oil administered quantita-

TABLE II.

*Reproduction Record of Females on Skimmed Milk Powder Diets.*

Ration No.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	days	days							per cent	
838	131	90	3	1	10	1	0	0	0	0.5 cc. cod liver oil per animal per day. Only one litter of young born; out of 10 young 9 delivered dead.
839	131	90	3	0	0	0	0	0	0	0.5 cc. cod liver oil per animal per day; increased at breeding to 0.7 cc. per animal per day.
840	130	90	3	1	2	0	0	0	0	0.5 cc. cod liver oil per animal per day; increased at breeding to 1.0 cc. per animal per day. Only one litter of 2 dead young found. Another litter born unseen.
837	272	220	3	9	79	71	47	34	72	0.2 cc. cod liver oil per animal per day; 3.0 per cent wheat oil in ration.
836	272	221	3	5	37	37	26	14	54	0.1 cc. cod liver oil per animal per day; 3.0 per cent wheat oil in ration.
835	272	221	3	6	46	46	35	15	43	3.0 cc. wheat oil in ration. All fat-soluble vitamins A and D derived from skimmed milk powder of ration and what traces might have been furnished by wheat oil in diet.

tively with a pipette in graduated amounts daily to each animal. (2) A synthetic diet containing large amounts of defatted wheat embryo as a source of vitamin B. (3) A highly purified synthetic diet, containing 5 per cent Harris yeast as a source of vitamin B.



*Detailed Reproduction Record of Females on Rations 835, 836, and 837.*

Ration No.	Female No.	Litter.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
							per cent	
837	3704	1st	11	11	6	6	100	Young found scattered on screen and partly eaten up.
		2nd	9	9	6	6	100	
		3rd	8	8	6	6	100	
		4th	5	5	5	0	0	
	3705	1st	11	11	6	6	100	All the young disposed of in few days by mother.
		2nd	10	10	6	0	0	
		3rd	8	8	6	6	100	
		4th	9	9	6	4	66	
	3706	1st	8	0	0	0	0	Litter of 8 young born dead; female in such poor physical condition that it was eliminated. Unfortunately record of continuous fertility and efficiency of lactation could not be secured on this animal.
836	3699	1st	9	9	6	0	0	
		2nd	7	7	6	6	100	
	3700	1st	10	10	6	6	100	
		2nd	2	2	2	2	100	
	3701	1st	9	9	6	0	0	
835	3694	1st	5	5	5	0	0	
		2nd	9	9	6	3	50	
		3rd	6	6	6	0	0	
	3696	1st	9	9	6	6	100	
		2nd	9	9	6	0	0	
		3rd	8	8	6	6	100	
	3695		0	0	0	0	0	

Holmes (8) has repeatedly demonstrated that, if any grade of cod liver oil is at all potent in vitamin A, 1 to 2 mg. per animal per day ought to more than suffice for preventing typical symptoms of xerophthalmia and at the same time induce growth. Hess<sup>2</sup> finds that 7 mg. of Mead Johnson and Company cod liver oil daily to each animal will bring about evidences of healing of rickets in a young rat which is receiving the Sherman-Pappenheimer ration. Table II clearly shows that 1.0 cc., or approximately 920 mg., of our cod liver oil is ineffectual in preventing sterility. During a reproduction period of 90 days two litters were born to two females out of nine, receiving 0.5 to 1.00 cc. of cod liver oil daily; one female bore nine dead young, and the other two dead young. Neither of these females produced second litters.

The results of experiments on Rations 835, 836, and 837 conclusively show the striking differences in reproduction obtained by the addition of wheat oil to the basal skimmed milk reproduction-deficient diet. The animals on Ration 835 received all of their fat-soluble vitamins A and D from 50 per cent skimmed milk powder and what traces might be contained in the 3 per cent wheat oil. Two females out of three gave birth to first, second, and third litters each, and succeeded in weaning fifteen young out of thirty-five allowed to be reared. Although we were already acquainted with the fact that our Stock Diet 1,<sup>3</sup> containing 1 per cent cod liver oil, produces considerable storage of fat-soluble vitamins in our experimental animals transferred from mothers on such a diet, we were very much surprised to find the continuous fertility performance and notable success in lactation of the females on Ration 835. This led us to investigate the vitamin A content of our wheat oil. Employing McCollum's vitamin A-deficient diet of rolled oats 40, casein 5,<sup>4</sup> NaCl 1.0, CaCO<sub>3</sub> 1.5, and dextrin 52.5, and a similar diet in which 3 per cent dextrin was replaced by 3 per cent wheat oil, both our control and experimental animals reached a maintenance curve in 43 days. Maintenance persisted for another month after which period xerophthalmia developed in

<sup>2</sup> Personal communication.

<sup>3</sup> Composition of Stock Diet 1: whole wheat, 27.0; rolled oats, 26.0; yellow corn, 25.0; oil meal, 15.0; commercial casein, 5.0; cod liver oil, 1.0; NaCl, 0.5; CaCO<sub>3</sub>, 0.5; and a liberal supply of whole milk.

<sup>4</sup> Free from fat-soluble vitamins.

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both control and experimental groups. Irradiation at this point with a mercury quartz vapor lamp for several days 20 to 30 minutes daily at a distance of 24 inches, brought no response in growth. This experiment demonstrates that there is considerable storage on our Stock Diet 1. Also, employing McCollum's

TABLE IV.

*Reproduction Record of Females on Synthetic Diets Containing Defatted Wheat Embryo as Source of Vitamin B.*

Ration No.	Duration of experiment.		Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be resed.	Young weaned.		Remarks.
	days	days								per cent	
867	118	74	3	1	4	4	4	4	0	0	0.3 cc. cod liver oil per animal per day. 30 per cent defatted wheat embryo as only source of vitamin B in ration. Two females resorbed first litters as determined by postmortem examination. Third female had only first litter of 4 young, disposed of in few days by mother.
868	244	204	3	10	83	83	52	50	96		0.3 cc. cod liver oil per animal per day. 30 per cent defatted wheat embryo and 3 per cent wheat oil in ration.

Note the difference in success of reproduction due to the presence of wheat oil in the ration.

method of biological assay of vitamin A, we have found no detectable amounts of such vitamin in the wheat oil employed in our vitamin E studies. We are studying this problem further on young animals coming from another rat colony which is receiving a different type of stock diet permitting of less storage of fat-

TABLE V.

*Reproduction Record of Females on Synthetic Diets Containing 5 Per Cent Harris Yeast as Source of Vitamin B, and Graduated Amounts of Cod Liver Oil, Administered Separately from Ration, as Only Source of Vitamin E.*

Ration No.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	days	days							per cent	
956	124	54	3	0	0	0	0	0	0	0.1 cc. cod liver oil per animal per day. Excellent growth.
957	124	54	2	1	6	1	0	0	0	0.3 cc. cod liver oil per animal per day. One male and one female failed in growth. Two females used for reproduction studies.
958	124	54	2	2	11	7	6	0	0	0.5 cc. cod liver oil per animal per day. One female had 5 young, 4 born dead; remaining 1 died next day. Other female had litter of 6 which disappeared 2nd day. Very good growth obtained in this experiment.
959	88	18	3	0	0	0	0	0	0	0.5 cc. cod liver oil per animal per day; increased at breeding to 1.00 cc. per animal per day. Complete failure in growth, most marked after mating.

soluble vitamins A and D. The vitamin D potency of our wheat oil is still to be demonstrated.

Tables II and III also show clearly that, in the presence of vitamin E, the beneficial effects of cod liver oil on fertility and lactation become apparent.

Table IV shows the results of two clean cut experiments which conclusively demonstrate the deficiency of our cod liver oil in the reproductive factor. Ration 868 is an exact duplicate of Ration 867, the only difference being that in the former 3 per cent wheat oil (prepared by percolating wheat germ with cold acetone) replaced an equivalent amount of dextrin in the diet. All the animals in both groups on the two types of rations received 0.3 cc. of cod liver oil (per animal per day) separately from the ration. On Ration 867 two females resorbed their first litters (determined by postmortem examination) and the third female gave birth to a first litter only of four young. On Ration 868 three females, during a reproduction period of 204 days, gave birth to ten litters (first, second, and third litters) of 83 young, all born alive, vigorous, and healthy, and out of 52 young allowed to be reared, 50 were successfully weaned, showing a lactation efficiency index of 96 per cent.

In connection with studies of reproduction on synthetic diets, employing Harris yeast as the only source of vitamin B we have for the last 2 years repeatedly observed the loss of potency of this commercial concentrated yeast product, which is supposed to be the Osborne and Wakeman concentrated vitamin B fraction from brewers' yeast (9). Although such yeast was inadequate for growth, at a 3 per cent level we have been able to secure very good, although not optimum growth, when this yeast was fed at a 5 per cent plane of intake. The same diet in every respect was fed to four groups of animals, but the cod liver oil was administered in graduated amounts daily to each animal. An examination of Table V shows that two facts stand out prominently as a result of this experiment. First, that cod liver oil as the only source of fat-soluble vitamins proved an absolute failure for fertility, and, second, that excessive amounts of cod liver oil supplementing the basal synthetic diet, containing Harris yeast, are followed by toxic effects on the growing organism, especially after mating.

#### SUMMARY.

1. The deficiency of cod liver oil, employed in this laboratory since 1921, in vitamin E has been demonstrated on three different types of diets.

2. This grade of cod liver oil is abundant in known fat-soluble vitamins other than E.

3. The same grade of cod liver oil administered in excessive amounts to animals receiving a highly purified synthetic diet, containing 5 per cent Harris yeast, is followed by toxic effects on growing animals, especially after mating.

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## **DIETARY REQUIREMENTS FOR REPRODUCTION.**

### **X. VITAMIN B REQUIREMENTS FOR NORMAL LACTATION.\***

By BARNETT SURE.

WITH THE TECHNICAL ASSISTANCE OF H. M. BOGGS.

*(From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville.)*

(Received for publication, March 23, 1927.)

The physiological functions claimed for vitamin B are many, and work of the last few years points to the fact that this syndrome is a complex composed of several chemical entities. As early as 1919 Mitchell (1) suggested that the antineuritic and growth-promoting substances may not be identical. Recently Hauge and Carrick (2) have presented experimental evidence supporting Mitchell's view. They have shown that the antineuritic substance when supplied in abundance will not promote rapid growth and that a diet may be capable of promoting rapid growth without preventing polyneuritis.

In 1925 McCollum, Simmonds, and Becker (3) showed that yeast, when serving as the sole source of vitamin B, was decidedly less effective than equal amounts of wheat germ in protecting rats against an ophthalmia produced by lack of mineral balance in the diet. The conclusion these investigators draw from their experiment is that vitamin B is not one substance but contains two or more principles. That vitamin B furnished by concentrated preparations from brewers' yeast controls gastric tonicity which in turn influences the appetite has recently been clearly demonstrated by Cowgill and coworkers (4). Smith and Hendrick (5) believe that brewers' yeast, generally accepted as a potent source of only one vitamin, contains some essential factor

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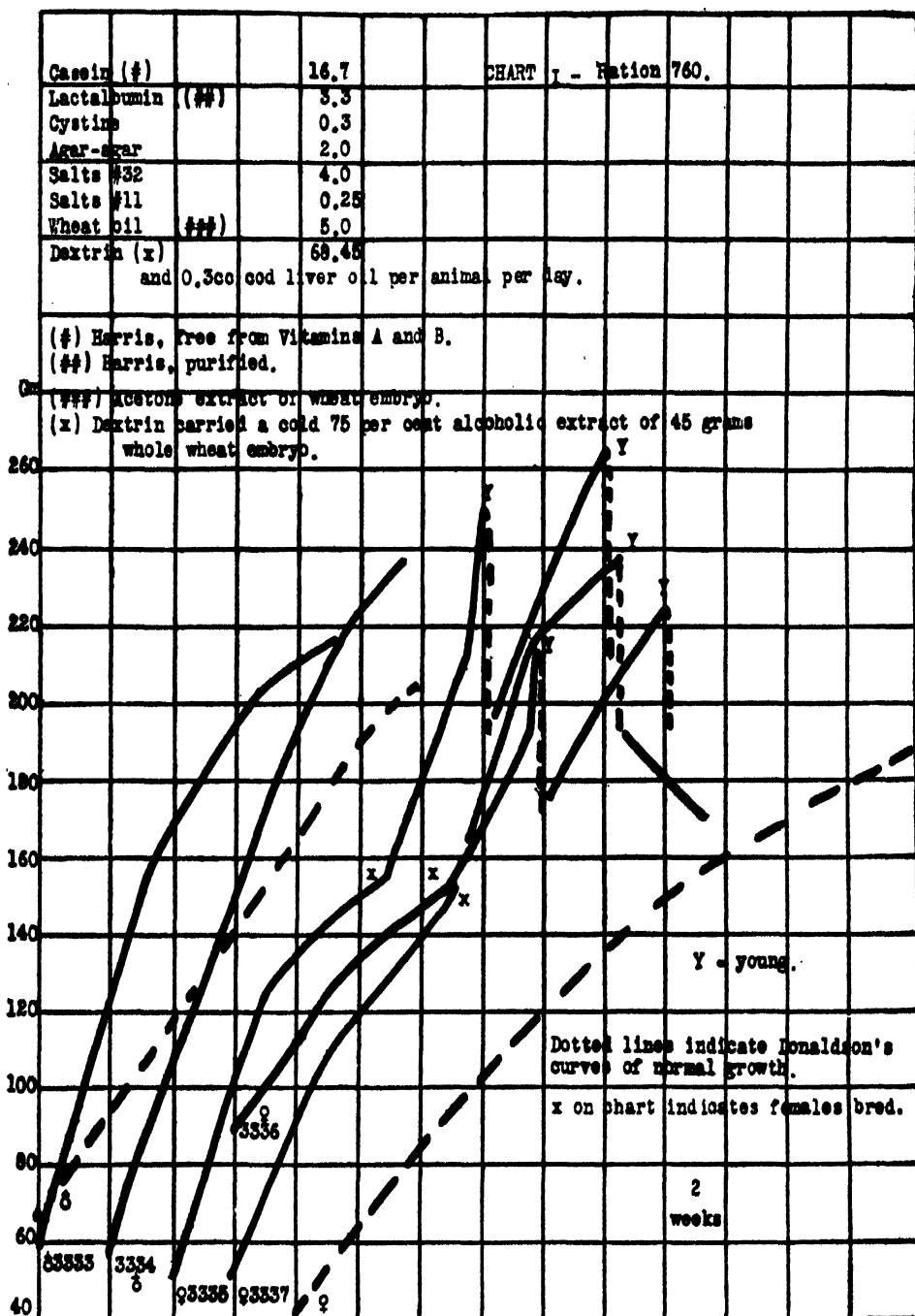


CHART I.

in nutrition other than vitamin B. The most recent contribution that Goldberger and coworkers (6) have made in their nutritional studies of pellagra is that Harris yeast, which is supposed to be the Osborne and Wakeman concentrated fraction from brewers' yeast, although deficient in the antiberiberi complex, is the most potent source they have as yet found for the cure of pellagra.

In this communication evidence is presented showing that alcoholic extracts of the wheat embryo, in addition to promoting growth and preventing beriberi in the rat, also exert a specific influence on lactation, and that the requirements of such extracts for normal mammary gland function are considerably greater than that for optimum growth.

The synthetic rations first employed in this investigation were made to approximate the composition of the proteins of milk as much as possible (see Chart I). Later, after finding that the character of the proteins in the synthetic diets was not the limiting factor responsible for the infant mortality, as determined by curative and preventive methods, the rations were simplified so that they contained casein as the only source of protein.

#### *Preparation of Alcoholic Extracts of Wheat Embryo.<sup>1</sup>*

*Cold 75 Per Cent Alcoholic Extract.*—100 pounds of whole wheat embryo were percolated with 60 gallons of 75 per cent ethyl alcohol at room temperature. The alcohol was evaporated *in vacuo* and any insoluble matter filtered. The yield of extract was generally about 10 per cent.

*Cold 25 Per Cent Alcoholic Extract.*—100 pounds of defatted wheat embryo (fat removed by percolation with acetone at room temperature, leaving about 0.1 per cent residual fat in embryo) were percolated with 60 gallons of 25 per cent ethyl alcohol at room temperature. The extracts were evaporated *in vacuo* to a syrup, and the yield was 40 per cent.

<sup>1</sup> The preparation of the wheat embryo extracts was carried out by Mr. E. H. Stuart, chemist of the Research Laboratories of Eli Lilly and Company, Indianapolis. Mr. Stuart used a wheat germ product secured in the vicinity of Indianapolis.

*Preparation of Acidulated Aqueous Extract of Wheat Embryo.*

100 pounds of whole wheat embryo were defatted with acetone, removed from percolator to pans, and air-dried to remove the acetone. They were packed again in the percolator, using to 10 gallons of water 100 cc. of glacial acetic acid, and 100 cc. of chloroform. 55 gallons of such acidulated water were used. The extract was heated to 75°C., 300 cc. of 17 per cent ammonia were added, and then it was filtered. The filtrate, slightly acid to litmus, was evaporated *in vacuo* to a syrupy consistency. 1 pound of extract was obtained from 6.6 pounds of original wheat embryo.

All the extracts were preserved by solution in 10 per cent ethyl alcohol. In the early experiments when 75 per cent ethyl alcoholic extracts of 45 gm. of whole wheat embryo per 100 gm. of ration were employed, they were poured directly onto the ration, thoroughly mixed by hand, and the ration was then ground in a small electric mill three to four times. By the time the ration reached the experimental animals the amount of alcohol carried by the extracts was negligible; at least, there were no noticeable symptoms on the rats. It was calculated from food consumption records that such extracts furnished the equivalent of 6.5 gm. of wheat germ per animal per day to growing rats consuming 14 gm. of ration daily. In our vitamin B studies we have found that food consumption during the nursing period, compared with periods preceding and following lactation<sup>2</sup> is approximately doubled; in other words, a lactating rat will consume instead of 14 gm. about 28 gm. of feed daily. Although a cold 75 per cent alcoholic extract of 6.5 gm. of whole wheat embryo will permit optimum growth in a mature rat, the same extract of as much as 11.2 gm. of embryo per animal per day, as a source of vitamin B, is still entirely inadequate for normal rearing of young. It was calculated from food consumption records that it is necessary to supply the same extract of as much as 22.4 gm. of whole wheat germ per animal per day to allow normal lactation. On the other hand, a 25 per cent alcoholic extract of approximately 13.0 gm. of whole wheat embryo was found sufficient per nursing rat per day for normal rearing and weaning of young.

<sup>2</sup> Unpublished data.

All 75 per cent alcoholic percolates were spread on dextrin and dried overnight at 70–80°C. in an electric oven; thus the

TABLE I.  
*Composition of Rations.*

0.3 cc. of cod liver oil was administered daily to each animal separately from the ration.

Ration No.	Casein.*	Casein.†	Lactalbumin.‡	Cytine.	Agar-agar.	Salts 32.	Ferric citrate.	Salts 11.	Wheat oil.§	Dextrin.
759		16.7	3.3	0.3	2.0	4.0		0.25	0.0	73.45
760		16.7	3.3	0.3	2.0	4.0		0.25	5.0	68.45
761		16.7	3.3	0.0	2.0	4.0		0.25	5.0	68.75
762		20.0	0.0	0.0	2.0	4.0		0.25	5.0	68.75
763		20.0	0.0	0.3	2.0	4.0		0.25	5.0	68.45
764		25.0	0.0	0.0	2.0	4.0		0.25	5.0	63.75
769		16.7	3.3	0.3	2.0	4.0		0.25	3.0	70.45
771		16.7	3.3	0.3	2.0	4.0		0.0	5.0	68.70
772		16.7	3.3	0.3	2.0	4.0	0.25	0.0	5.0	68.45
796	25.0				2.0	4.0		0.25	5.0	63.75
797	20.0				2.0	4.0		0.25	5.0	68.75
798	16.7		3.3	0.0	2.0	4.0		0.25	5.0	68.75
799	16.7		3.3	0.3	2.0	4.0		0.25	5.0	68.45
800	16.7		3.3	0.3	2.0	4.0		0.25	0.0	73.45¶
879		20.0			2.0	4.0	0.20		0.0	73.80**
880		20.0			2.0	4.0	0.20		0.0	73.80††

\* Commercial casein.

† Harris, free from vitamins A and B.

‡ Harris, purified.

§ Acetone extract of wheat embryo.

|| Carried a cold 75 per cent alcoholic extract of 45 gm. of whole wheat embryo per 100 gm. of ration.

¶ Carried a cold 75 per cent alcoholic extract of 80 gm. of whole wheat embryo per 100 gm. of ration.

\*\* Carried a cold 25 per cent alcoholic extract of 40 gm. of acetone-extracted wheat embryo per 100 gm. of ration.

†† Carried an acidulated aqueous extract of 40 gm. of acetone-extracted wheat embryo per 100 gm. of ration.

dextrin carried the vitamin B in the rations. The acidulated aqueous extracts and the 25 per cent alcoholic extracts were also spread on dextrin and subjected to preliminary drying in the air

TABLE II.  
*Lactation Record of Animals on Rations Described in Table I.*

Ration No.	Dura- tion of experi- ment.	Repro- duction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	days	days							per cent	
759	142	95	3	3	6	4	4	0	0	Control experiment on vitamin E. Female 3330 had first litter of 2 young and resorbed second litter. Female 3331 had first litter of 4 young, which it dis- posed of in few days; resorbed second litter. Female 3332 had first litter of 2 young; resorbed second litter. Resorptions determined by postmortem ex- amination.
800	110	72	3	1	3	1	0	0	0	Control experiment on vitamin E. Ration contained amount of vitamin B which in presence of vitamin E would have been adequate for fertility and also lactation.
760	160	112	3	6	34	34	28	4	14	Two females had first litters of 6 and 4 young; both disposed of young in few days. On second mating ration changed so that dextrin carried 75 per cent alcoholic extract of 60 instead of 45 gm. whole wheat embryo. Three second litters of 8, 5, and 6 young. Out of total of 17 allowed to be reared, only 4 suc- cessfully weaned.
761	104	61	3	3	21	19	18	4	22	During latter part of pregnancy vitamin B content of ration increased so that dextrin carried alcoholic extract of 60 instead of 45 gm. whole wheat embryo.

762	77	25	2	2	12	12	11	0	0	Both litters disposed of by mother on 2nd day of lactation.
763	100	40	3	3	24	24	18	0	0	
764	84	28	3	3	30	29	18	0	0	On day of birth vitamin B content of ration increased so that dextrin carried alcoholic extract of 60 instead of 45 gm. whole wheat embryo. Two litters died during 1st week of lactation; third litter nursed by mother for 2 weeks, then succumbed.
769	129	79	3	3	28	28	18	17	94	On day of birth ration changed so that dextrin carried alcoholic extract of 60 instead of 45 gm. whole wheat embryo. Three first litters. All young died on 2nd day after delivery. Detailed lactation record shown in Table III. Two changes made in vitamin B content of diet so that ultimately ration contained alcoholic extract of 80 gm. whole wheat embryo.
771	77	26	2	2	18	18	18	0	0	Both litters died on 2nd day of lactation.
772	130	80	3	3	23	23	18	18	100	During latter part of pregnancy ration changed so that dextrin carried alcoholic extract of 80 instead of 45 gm. whole wheat embryo.
796	97	56	3	2	14	14	11	0	0	The females bred on May 4. On May 29 the vitamin content of ration increased so that dextrin carried alcoholic extract of 60 gm. whole wheat embryo. One litter of 8 young born May 26; another litter of 9 June 8. Both litters successfully reared until the 15th to 16th day of lactation; were then gradually reduced so that all young died within succeeding 5 days.

Ration No.	Dura- tion of experi- ment.	Repro- duction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared	Young weaned.		Remarks.
	days	days							per cent	
797	126	84	3	3	32	32	18	18	100	Detailed lactation record shown in Table IV. Two changes made in vitamin B content of diet, so that ultimately ration contained alcoholic extract of 80 gm. whole wheat embryo.
798	101	48	2	2	18	18	12	0	0	During latter part of lactation vitamin B content of ration increased so that dextrin carried alcoholic extract of 60 instead of 45 gm. whole wheat embryo. Both litters died during latter part of lactation.
799	119	71	3	3	22	22	18	12	66	Two changes made in vitamin B content of diet so that ration ultimately contained alcoholic extract of 80 gm. whole wheat embryo.

TABLE III.

*Lactation Record of Females 3380, 3381, and 3382, Ration 769.*

Dextrin carried a cold 75 per cent alcoholic extract of 45 gm. of whole wheat embryo per 100 gm. of ration.

Date.	Fe- male 3380.	Litter.	Fe- male 3381.	Litter.	Female 3382.	Litter.	Date.	Female 3381.	Litter.
<i>May</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>June</i>	<i>gm.</i>	<i>gm.</i>
7	222		154		191		16		155
8	230				194		17		158
9	167	31 (6)*			200		19		164
10		31			206		20		180
11	160	37			218		21		184
12†		41	164		228		22		190
13		44			240		24		205
14		47			184	30 (6)*	26		213
15	165	49			33		27		214
16	167	51	175		192	38	28		214
17		56	181		192	41	29		212
18	170	60	189		192	47	30	166	178 (5)*
20	170	72	200		180	60	<i>July</i>		
21	168	78	202		183	66	1		192
22	162	83	203		180	73	2		204†
23	160	86	205		179	80	3		220
24	156	89	151	28 (6)*	174	85			
25		95	148	30	178	93			
27		108		38		110			
29	166	120		50		125			
30		127		54		135			
<i>June</i>									
2		144		71		161			
4		158		83		174			
6		176		99		192			
8		200		112		212			
10		215		125	176	242†			
12		230		140					
13	178	242†		143					
14	173	255		147					
15				150					

Females 3380 and 3382 were bred April 15 and had litters of nine and eleven young respectively. Female 3381 was bred May 1st and had a litter of eight young.

\* Number of young on date indicated.

† On May 12 ration was changed, so that dextrin carried an alcoholic extract of 60 gm. of whole wheat embryo, and on May 24 the vitamin B content of the ration was further increased, so that dextrin carried an alcoholic extract of 80 gm. of whole wheat embryo.

‡ Weaned.



TABLE IV.

*Lactation Record of Females 3520, 3521, and 3522, Ration 797.*

Dextrin carried a cold 75 per cent alcoholic extract of 45 gm. of whole wheat embryo per 100 gm. of ration.

Date.	Female 3520.	Litter.	Female 3521.	Litter.	Female 3522.	Litter.	Date.	Female 3520.	Litter.
<i>May</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>July</i>	<i>gm.</i>	<i>gm.</i>
24	179		237		148				
27	192		261				8		77
28	195		263				9		86
29*	188		199	30 (6)†			11		105
30	187			31			12		118
31	196			38	171		14		132
<i>June</i>							16		150
1				40			18		166
2				47			20		179
4	200			61	197		22		193
5	200			70	206		24		210
6				80	156	22 (6)†	26		232
7				88		25	27	224	244‡
9				102		33			
10				108		38			
11	204			115		44			
12	201		172	112		48			
13			160	103		53			
14				108		60			
15				118		67			
16				128		74			
17				133		81			
19	218			156		104			
21				173		113			
22	222			180		120			
24	231			197		132			
25	238			205		138			
26*	236		197	199		142			
27	254			228		150			
28	271		197	244‡		158			
29	274		192	246		166			
30	284		197	258		173			
<i>July</i>									
1	218	30 (6)†		274		186			
2		33				200			
3		40				212			

TABLE IV—*Concluded.*

Date.	Fe- male 3520.	Litter.	Female 3521.	Litter.	Female 3522.	Litter.	Date.	Female 3520.	Litter.
<i>July</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>July</i>	<i>gm.</i>	<i>gm.</i>
4		45				223			
5		52				232			
6		60				238			
7		70				247†			

Females 3520 and 3521 were bred on May 4 and had litters of ten and eleven young respectively. Female 3522 was bred on May 21 and had a litter of eleven young.

\* On May 29 the vitamin B content of the ration was increased, so that dextrin carried an alcoholic extract of 60 instead of 45 gm. of whole wheat embryo, and on June 26 the vitamin B concentration of the ration was further increased, so that dextrin carried an alcoholic extract of 80 gm. of whole wheat embryo.

† Number of young allowed to be reared.

‡ Weaned.

by aid of an electric fan for 24 hours. To complete the drying, so that the dextrin carrying the syrupy vitamin B preparations could be successfully mixed with our synthetic diets, it was necessary to heat these extracts overnight in the electric oven at temperatures of 100–105°C. There are no facilities for vacuum drying in this laboratory. It is quite possible that by such drastic heat treatment there occurred some vitamin destruction. All the biological experiments on lactation and growth were, however, conducted with extracts prepared by the same laboratory procedure, and the results are considered strictly comparable.

The results of the work on lactation are shown in Tables II to VI inclusive in such detail that very little restatement or discussion is necessary. It is clear from Tables I and II that, regardless of the nature of the amino acid content of the diet, when cold 75 per cent alcoholic percolates of 45 gm. of whole wheat embryo per 100 gm. of ration serve as the only source of vitamin B, while growth proceeds at an optimum rate observed in our colony on the best of stock diets, lactation is an absolute failure. Increase of such extracts, so that each 100 gm. of ration contain the equivalent of 60 gm. of embryo, is followed by improvement in rearing of young, although considerable infant mortality is

TABLE V.

*Record of Two Females (First Litters) Transferred from Stock Diet 1 during Latter Part of Pregnancy and Placed on Experimental Diet 880 (See Table I); 0.8 Cc. of Cod Liver Oil Allowed Daily to Each Female.*

Dextrin carried an acidulated aqueous extract of 40 gm. of acetone-extracted wheat embryo per 100 gm. of ration.

Date.	Female 3901.	Litter.	Food consumed during previous 24 hrs.	Female 3902.	Litter.	Food consumed during previous 24 hrs.	Remarks.
July	gm.	gm.	gm.	gm.	gm.	gm.	
23	312			334			Females 3901 and 3902 had litters of 14 and 12 young respectively, and were each allowed 9 to nurse.
24	306		0	320		15	
25	306		0	308		5	
26	210	39 (9)*	0	228	40 (9)*	0	
27		38	8		40	0	
28		44	14		45	29	
29		52	24	226	47	1	
30		65	13		56	8	
31		72	11		63	19	
Aug.							
1		78	14		70	4	0.5 cc. wheat oil† to each female. 0.5 " " " " " " 0.5 " " " " " "
2		88	21		80	9	
3	202	98	19	221	90	11	
4	200	106	18	213	96	27	
5	194	113	18	197	98	8	
6	193	121	22	208	105	24	
7	194	126	18	206	110	16	
8	199	130	27	203	114	22	
9	196	138	25	201	118	18	
10	192	142	22	202	120	20	
11	192	146	26	195	122	13	
12	190	150	23	192	123	7	0.5 cc. wheat oil† to each female. 0.5 " " " " " "
13	190	152	23	189	125	29	
14	190	156	26	191	130	23	
15	184	162	19	184	133	17	
16	180	169	21	185	138	19	
17	174	171	18	181	140	18	
18	173	172	21	175	140	16	
19	174	173	19	177	146	16	

\* No. of young allowed to be reared.

† The wheat oil was prepared in our laboratory by extracting wheat embryo with ether.

TABLE VI.

*Record of Two Females (First Litters) Transferred from Stock Diet 1 during Latter Part of Pregnancy and Placed on Experimental Diet 879 (See Table I); 0.3 Cc. of Cod Liver Oil Administered Daily to Each Female.*

Dextrin carried a cold 25 per cent alcoholic extract of 40 gm. of acetone-extracted wheat embryo per 100 gm. of ration.

Date.	Female 3899.	Litter.	Food consumed during previous 24 hrs.	Female 3900.	Litter.	Food consumed during previous 24 hrs.
<i>July</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
23	310			272		
24	246	42 (9) *	0	273		20
25		46	6	280		12
26		48	16	275		5
27		52	18	205	38 (9) *	5
28		64	18		45	10
29		72	22		51	22
30		80 (8) †	16		62	18
31		90	18		70	21
<i>Aug.</i>						
1		102	21		80	15
2		111	25		92	28
3	226	122	26	208	105	30
4	224	133	29	204	114	26
5	225	142	25	200	124	24
6	218	153	29	200	137	30
7	220	159	24	198	147	22
8	221	168	29	202	156	32
9	223	182	34	202	167	36
10	225	191	38	196	176	32
11	214	202	26	198	184	32
12	217	207	30	197	192	31
13	223	217	32	204	195	30
14	221	235	34	200	202	43
15	220	251	31	198	211	31
16	223	236	33	198	227	33
17	219	247	32	199	238	38
18	210	253	34	191	246	34
19	213	270	36	194	263	42
20	220	293	50 †	199	286	48 ‡
21	230	322	58	208	312	60
22	224	337	64	206	322	60
23	226	350	46	206	352	57
24	217	366	56	198	362	55

\* Females 3899 and 3900 each had litters of thirteen young and were each allowed nine to rear.

† Young left on date indicated.

‡ Young of both females began eating.

still encountered; and further increase of the same extracts, so that 100 gm. of ration carry the equivalent of 80 gm. of embryo, results in remarkable success in lactation. For the first time in 7 years we have observed lactating rats on purified synthetic diets nurse one litter after another and successfully wean the litters allowed to be reared, and this has occurred only among such animals as received rations containing high levels of alcoholic extracts of wheat embryo as the only source of vitamin B. It will be noted that all the rations (Table I) on which this notable success in lactation was secured contained also 5 per cent wheat oil as a source of vitamin E. On Ration 759, one of our control diets containing adequate proportions of vitamin B, but deficient in vitamin E, only first litters, disposed of by the mothers early in lactation, were obtained. The number of young was few, ranging from two to four per litter, and the few young born were either dead or were in an emaciated condition at birth.

Tables II to VI unmistakably show that the 25 per cent alcoholic extracts are the most potent for lactation. The objection to these extracts is that they do not represent a concentrated fraction (the yield being 40 per cent) and they are of a syrupy consistency too cumbersome to handle in the diet. The acidulated aqueous extracts were much inferior in potency to the 25 per cent alcoholic extracts for lactation.

Concerning the toxicity of wheat embryo, postulated in 1916 by McCollum, Simmonds, and Pitz (7), we have found, from studies in this laboratory carried on during the past 4 years with ether and acetone extracts of wheat germ, different results. On synthetic diets containing as much as 5 per cent of wheat oil we have not only secured excellent growth far superior to that reported by Donaldson but have also succeeded in rearing and weaning over 150 young at a normal rate. One batch of wheat oil, prepared in the laboratories of Eli Lilly and Company, proved toxic at a 5 per cent level. Three females began to fail rapidly in growth at the age of 60 days on a synthetic diet containing 5 per cent of acetone extracts of wheat embryo. Reduction of the concentration of wheat oil to 3 per cent was immediately followed by a resumption of growth. Normal growth is a record which all of our animals must satisfy before they are eligible for lactation studies, and such growth we have secured on our synthetic

rations containing 3 to 5 per cent wheat oil, on which studies in rearing of young have been conducted.

#### SUMMARY.

1. A cold 75 per cent ethyl alcoholic extract of 6.5 gm. of whole wheat embryo (secured from Indianapolis mills) per animal per day furnishes enough vitamin B for excellent growth. It is necessary, however, to supply the same alcoholic extract of at least 22.4 gm. of wheat germ per lactating rat per day to furnish sufficient vitamin B for normal lactation. A cold 25 per cent ethyl alcoholic extract of approximately 13.0 gm. of the same wheat embryo per nursing animal per day furnishes sufficient vitamin B for normal rearing and weaning of young.

2. The requirements of vitamin B for normal mammary gland function are considerably greater than that for optimum growth.

3. Wheat oil fed at 3 and 5 per cent levels in our rations was found to be non-toxic to the rat.

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## DIETARY REQUIREMENTS FOR REPRODUCTION.

### XI. THE POTENCY OF BUTTER FAT IN VITAMIN E.\*

BY BARNETT SURE.

WITH THE TECHNICAL ASSISTANCE OF H. M. BOGGS.

(From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville.)

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During the last few years conflicting reports have been forthcoming concerning the biological value of butter fat as the only source of fat-soluble vitamins for reproduction. The literature on this subject has been recently thoroughly reviewed in this *Journal* by Mattill and Clayton (1).

Since 1923 Evans and coworkers have published no detailed experimental data on their studies of nutrition and fertility, and it is therefore difficult to know to what extent they may have modified their view on butter fat as a source of the reproductive factor. Indeed, two recent statements of the California investigators seem conflicting. Evans, in his Mayo Foundation Lecture on the "Relations between Fertility and Nutrition" states: "We have found, for instance, that when lard is omitted from the diet, 5 per cent butter fat often suffices to confer fertility on the animals throughout the early portion, and in some cases the greater portion of the life span." In the recent report of Evans and Burr (2) before the National Academy of Sciences on the "Antisterility Vitamin Fat-Soluble E" the following statement appears: "E is present but extremely low in milk fat. 9 per cent of this, which is included in our basic ration, together with 15 per cent lard, fails to prevent sterility, though with lard absent, 24 per cent succeeds. Whole milk powder may constitute one-third of the ration by weight and sterility result." According to the latter statement,

\* Research paper No. 47, Journal Series, University of Arkansas.



## 72 Dietary Requirements for Reproduction. XI

*Evans and Burr have not departed from the original view of Evans and Bishop concerning the vitamin E potency of milk fat (3).*

TABLE I.  
*Composition of Skimmed Milk Powder Diets.*

Ration No.	Skimmed milk powder.	Agar-agar.	Ferric citrate.	Salts II.	Harris yeast.*	Butter fat.	Cod liver oil.	Dextrin.	Remarks.
541	50.0	2.0		0.25	1.0	5.0	2.0	40.35	
542	50.0	2.0		0.25	1.0	3.5	2.0	41.85	
543	50.0	2.0		0.25	1.0	2.0	2.0	43.35	
544	50.0	2.0		0.25	1.0	1.0	2.0	44.35	
545	50.0	2.0		0.25	1.0	0.5	2.0	44.85	
598	50.0	2.0		0.25	1.0	7.0	2.0	38.35	
599	50.0	2.0		0.25	1.0	10.0	2.0	35.35	
682	50.0	2.0		0.25	1.0	0.0	0.0	47.35	0.3 cc. cod liver oil daily to each animal.
683	50.0	2.0		0.25	1.0	0.5	0.0	46.85	" "
684	50.0	2.0		0.25	1.0	1.0	0.0	46.35	" "
685	50.0	2.0		0.25	1.0	2.0	0.0	45.35	" "
686	50.0	2.0		0.25	1.0	3.0	0.0	44.35	" "
687	50.0	2.0		0.25	1.0	5.0	0.0	42.35	" "
689									
690	50.0	2.0		0.25	1.0	10.0	0.0	37.35	
691	50.0	2.0		0.25	1.0	5.0	0.0	42.35	
756	50.0	2.0	0.25		1.0	1.0	0.0	46.35	
757	50.0	2.0	0.25		1.0	2.0	0.0	45.35	
758	50.0	2.0	0.25		1.0	3.0	0.0	44.35	

\* From weaning time up to the beginning of the reproduction period rations contained 0.4 per cent Harris yeast. The increase was made at the time of mating and that percentage left in the rations until termination of the experiments.

The experiments reported in this communication have extended over a period of more than 2 years, and while some of this work is still in progress, enough data have now been accumulated to warrant its publication. As the investigation progressed it became

TABLE II.  
*Composition of Synthetic Diets.*

Ration No.	Casein.*	Casein.†	Lactalbumin.‡	Cystine.	Agar-agar.	Salts 32.	Salts 11.	Butter fat.	Wheat oil.	Cocoa butter.	Cotton seed oil.	Crude corn oil.	Peanut oil.	Defatted wheat embryo.	Dextrin.	Remarks.
777		16.7	3.3	0.3	2.0	4.0	0.25	1.0							72.45§	0.3 cc. cod liver oil daily to each animal.
778		16.7	3.3	0.3	2.0	4.0	0.25	2.0							71.45§	0.3 " " " "
779		16.7	3.3	0.3	2.0	4.0	0.25	3.0							70.45§	0.3 " " " "
780		16.7	3.3	0.3	2.0	4.0	0.25	5.0							68.45§	0.3 " " " "
913	20.0				2.0	4.0	0.25	5.0						30.0	38.75	0.3 " " " "
931	20.0				2.0	4.0	0.25	5.0						30.0	38.75	0.3 " " " "
914	20.0				2.0	4.0	0.25	5.0						30.0	38.75	All fat-soluble vitamins furnished by butter fat in ration.
930	20.0				2.0	4.0	0.25	5.0						30.0	38.75	
915	20.0				2.0	4.0	0.25	10.0						30.0	33.75	" " " "
932	20.0				2.0	4.0	0.25	10.0						30.0	33.75	" " " "
938	20.0				2.0	4.0	0.25		1.0					30.0	42.75	0.3 cc. cod liver oil daily to each animal.
940	20.0¶				0.0	4.0	0.25		1.0					30.0	44.75	0.3 " " " "
919	20.0				2.0	4.0	0.25			5.0				30.0	38.75	0.3 " " " "
916	20.0				2.0	4.0	0.25				5.0			30.0	38.75	0.3 " " " "
917	20.0				2.0	4.0	0.25					5.0		30.0	38.75	0.3 " " " "
918	20.0				2.0	4.0	0.25						5.0	30.0	38.75	0.3 " " " "

\* Washed with dilute acetic acid for a week Harris, free from vitamins A and B. † Harris, purified.  
 § Dextrin carried a cold 75 per cent alcoholic extract of 45 gm. of whole wheat embryo, as a source of vitamin B, per 100 gm. of ration.  
 || Acetone extract of wheat embryo. ¶ Commercial casein was used in this ration.

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TABLE III.

*Reproduction Record of Females Receiving Butter Fat Additions to Skimmed Milk Powder Diets. Cod Liver Oil in Ration.*

Ration No.	Butter fat.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	per cent	days	days							per cent	
545	0.5	159	82	2	0	0	0	0	0	0	On 42nd day after mating ration changed so that dextrin carried unsaponifiable matter from wheat oil equivalent to 1.0 per cent original oil in ration. During subsequent 50 days only one litter of 4 young was cast, 3 born dead and premature-looking.
544	1.0	159	78	3	0	0	0	0	0	0	
543	2.0	162	92	3	0	0	0	0	0	0	
542	3.5	151	81	3	0	0	0	0	0	0	None of the females on ration showed signs of advanced pregnancy after being bred for 41 days at which time ration was changed so that dextrin carried 0.1 per cent unsaponifiable matter from wheat oil in ration, equivalent to 3.0 per cent original oil. During the remainder of reproduction period three litters were born, consisting of 5, 9, and 4 young. None of litters weaned.
541	5.0	188	121	3	0	0	0	0	0	0	All young pale and emaciated and disposed of by mother in few days after birth.
598	7.0	88	32	3	3	16	13	11	0	0	

TABLE III—*Concluded.*

Ration No.	Butter fat.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	per cent	days	days							per cent	
599	10.0	102	45	3	3	23	17	13	0	0	One female attempted to rear 4 young for 23 days. On 23rd day litter collectively weighed 105 gm.; failed on following day.

absolutely essential to study reproduction from the standpoint of continuous fertility, and such findings are also submitted in this paper.

Table III shows the effect on reproduction of addition of graduated amounts of butter fat to a skimmed milk powder ration containing 2 per cent cod liver oil. The outcome is a positive failure. Note that the addition of small amounts of unsaponifiable matter from wheat oil to the same types of diets is followed by beneficial effects on fertility. The fact that these litters were not weaned was surprising, since such preparations of unsaponifiable matter from wheat oil added to the same types of diets on previous occasions resulted in appreciable success in lactation (4). Whether or not storage of the unsaponifiable matter, since it was kept for several months in a corked bottle, was a contributing factor in the loss of its lactation-promoting properties we have not determined.

The results of experiments on Rations 756, 757, and 758 (Table IV) when compared with findings reported in a preceding paper of this series, are interesting at this point.<sup>1</sup> The addition of 3 per cent butter fat to the basal skimmed milk powder diet, from which cod liver oil was removed, resulted in absolute failure in reproduction. The substitution of the 3 per cent butter fat by 3 per cent wheat oil was followed by appreciable success in fertility and lactation.<sup>1</sup> Two females each gave birth to first, second, and third litters. Forty-six young were born during a reproduction period

<sup>1</sup> See Paper IX in this issue.

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TABLE IV.

*Reproduction Record of Females Receiving Butter Fat Additions to Skimmed Milk Powder Diets. Cod Liver Oil Removed from Ration.*

Ration No.	Butter fat.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	per cent	days	days							per cent	
756	1.0	81	25	3	0	0	0	0	0	0	First litters. Two resorptions (postmortem examination). Third female showed no signs of advanced pregnancy during reproduction period.
757	2.0	77	21	3	0	0	0	0	0	0	Three resorptions (postmortem examination). Female 3317: 10 embryos; placenta full of water. Female 3316: 8 embryos. Female 3315: small remains of embryos.
758	3.0	118	58	3	0	0	0	0	0	0	Both males in experiment showed unmistakable external signs of atrophied testes. At second breeding replaced by stock males. No signs of advanced pregnancy 31 days thereafter.
682	0.0	83	32	3	2	7	6	6	0	0	Control experiment. 0.3 cc. cod liver oil daily to each animal. One female resorbed litter (postmortem examination). Another had only 1 young, born dead. Third female had 6 young, disposed of by mother in few days.

TABLE IV—*Concluded.*

Ration No.	Butter fat.	Duration of experiment.		Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.		Young weaned.		Remarks.
	per cent	days	days								per cent		
683	0.5	90	40	3	2	13	9	9	0	0			0.3 cc. cod liver oil daily to each rat. One female had a litter of 8 young, 4 born dead; another had 5 young; third showed resorption curve at termination of experiment.
684	1.0	135	83	3	1	6	6	6	0	0			0.3 cc. cod liver oil daily to each rat.
685	2.0	111	72	3	3	23	18	16	3	19			" "
686	3.0	110	71	3	3	18	18	14	0	0			" "
687	5.0	158	106	3	3	34	31	24	9	37			One female had litter of only 2 young. First and second litters. 0.3 cc. cod liver oil daily to each animal.
691	5.0	129	49	2	2	16	15	11	0	0			Experiment identical with No. 687 except animals received no cod liver oil.*
689	10.0	126	68	6	6	52	51	34	22	65			
690													

\* The interesting observation in this experiment was to see one female rear her litter of six young up to 17th day of lactation to a weight of 31 gm. each, quite normal at this period of nursing, then develop suddenly the most abnormal infanticidal characteristics. The author and attendant of the animals watched the rats kill each young, and then consume the internal organs and blood.

of 221 days, and out of thirty-five young allowed to be reared, fifteen were successfully weaned. The results of these experiments are so striking that no discussion is necessary to show the superiority of wheat oil to butter fat for purposes of reproduction.

Increasing the amount of butter fat from 5 to 10 per cent in the basal skimmed milk powder diet resulted in considerable success in fertility and lactation (Rations 689, 690, and 691, Table IV).

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The beneficial results obtained in fertility by removal of cod liver oil from the ration are also apparent (Ration 541, Table III; and Ration 686, Table IV). Such results, and those obtained by

TABLE V.

*Reproduction Record of Females Receiving Butter Fat Additions to Purified Synthetic Rations Containing Alcoholic Extracts of Wheat Embryo as Source of Vitamin B.*

0.3 cc. of cod liver was administered separately from the ration daily to each animal.

Ration No.	Butter fat.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	per cent	days	days							per cent	
759	0.0	142	90	3	3	8	6	6	0	0	Only first litters born. One female had 2 dead young, another 4 young, the third 2 young. Two females resorbed second litters; third showed no signs of advanced pregnancy from May 21 to July 8.
777	1.0	109	68	3	3	25	24	16	0	0	One female died 2nd day after parturition; another resorbed second litter.
778	2.0	79	39	3	2	18	18	12	0	0	One female sterile.
779	3.0	110	68	2	3	19	19	12	0	0	One female resorbed second litter; other had only 3 young in second litter.
780	5.0	138	69	3	2	9	4	4	0	0	None of the females showed any signs of advanced pregnancy after being rebred for 42 days.

Evans by removing lard from his basal synthetic diet, necessarily raise the question, has the vitamin E theory been postulated on account of dietary mismanagement? This question can only be most satisfactorily answered after the presentation of the reproduction data on synthetic diets.

On Ration 759 which is a control vitamin E-deficient diet, three females gave birth to eight young collectively. One female had two young delivered dead; another had four young; and the third had a litter of only two young. Two of these females resorbed their second litters, and the third showed no signs of advanced pregnancy for the third litter.

Table V shows the effect of additions of graduated amounts of butter fat, 1 to 5 per cent in the ration, to purified synthetic diets on reproduction. Only first litters were generally obtained. Second litters were resorbed as determined by postmortem examination. Only one female out of eleven had a second litter and the number of young in that was only three.

*Detailed Reproduction Records of Females Receiving Rations 777 to 780 Inclusive.*

*Ration 777.*—Female 3420 gave birth to a litter of nine young, six of which weighed 24 gm. On the 2nd day after delivery the mother was found dead. Female 3421 had a litter of nine young, and disposed of the six young allowed her to rear on the 2nd day. Female 3422 had seven young, three of which were born dead. The rest of the young were disposed of by the mother within 24 hours. The two remaining females were rebred, at which time the ration was changed so that dextrin carried 75 per cent alcoholic extracts of 80 gm. of whole wheat embryo. Female 3422 resorbed its second litter (postmortem examination) and female 3421 showed no signs of advanced pregnancy 47 days after second mating.

*Ration 778.*—Female 3425 had seven young, and Female 3427 had a litter of eleven young. Both litters were devoured by their mothers within a few days after delivery. One female showed no signs of advanced pregnancy after being bred for 6 weeks.

*Ration 779.*—One female failed in growth on this ration and was, therefore, eliminated. Female 3430 had a litter of six young and disposed of it on the 2nd day. Female 3432 had ten young, all of which were found dead on the 2nd day. When the two females were rebred the vitamin B content of the ration was increased, so that dextrin carried, instead of a 75 per cent alcoholic extract of 45 gm., the extract of 80 gm. of whole wheat embryo per 100 gm. of ration. Female 3432 had a second litter of only three young which it did not rear, and Female 3430 resorbed its second litter (postmortem examination).



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*Ration 780.*—Female 3435 showed no signs of advanced pregnancy throughout the entire reproduction period, which lasted 69 days. Female 3436 gave birth to a litter of six young, five of which were delivered dead. Female 3437 had a litter of only three young, all of which were found dead on 2nd day. On June 13, females were rebred, at which time the vitamin B content of the ration was increased, so that dextrin carried alcoholic extracts of 80 instead of 45 gm. of whole wheat embryo. On July 24 none of the females showed any signs of advanced pregnancy.

On a synthetic ration, containing 30 per cent defatted embryo (introducing only 0.03 per cent fat), supplemented by administration of 0.3 cc. of cod liver oil per animal per day, reproduction is a failure.<sup>1</sup> Out of three females, two resorbed their first litters and one had only a first litter of four young during a reproduction period of 74 days. We have since secured some additional data of a duplicate experiment. During a reproduction period of 95 days one female has as yet shown no advanced signs of pregnancy, another gave birth to one dead young, and the third to three dead young. On a similar ration, in which 3 per cent of the dextrin was replaced by 3 per cent wheat oil, during a reproduction period of 204 days, three females gave birth to ten litters of 83 young, all born alive, and out of 52 young allowed to be reared, 50 were successfully weaned, giving a lactation efficiency index of 96 per cent. Because of the pronounced success with first litters in fertility and lactation, the experiment was continued so as to secure data on continuous fertility. In this connection I agree with the interpretation of Mattill and Clayton (1) that, "Apparently enough vitamin E remains from the preexperimental weaning period to carry the animal through one gestation but not beyond one." Continuous fertility, then, must be adopted as the criterion for the potency of vitamin E-containing materials. Now, in the experiments just referred to, the cod liver oil, as a source of fat-soluble vitamins, was administered quantitatively by pipette in the mouths of the experimental animals, so all possible reaction of cod liver oil with the rest of the components of the diet (5) was entirely circumvented, and the argument that the vitamin E theory has been postulated on account of dietary mismanagement is entirely dispensed with.

In the series of experiments reported in Tables VI and VII the

same type of synthetic diets, containing 30 per cent of defatted wheat embryo as a source of vitamin B, have been employed as previously described.<sup>1</sup> The only change made is that Salts 11 (4) replaces an equivalent amount of ferric citrate in the ration. No injurious effects on fertility and lactation have been encountered after such modification.

TABLE VI.

*Reproduction Record of Females Receiving Butter Fat Additions to Synthetic Diets Containing Defatted Wheat Embryo as Source of Vitamin B.*

Ration No.	Duration of experiment.	Reproduction period.	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.		Remarks.
	days	days							per cent	
930	108	52	3	2	18	18	12	0	0	5.0 per cent butter fat in ration. One female died after being on experiment for 80 days. Two litters born to two females.
914	209	142	3	4	18	18	18	5	28	Same ration and experiment as No. 930.
913	209	160	3	9	77	61	39	2	5	5.0 per cent butter fat in ration, 0.3 cc. cod liver oil daily to each animal.
931	104	62	2	3	31	17	12	0	0	Same ration and experiment as No. 913.
932	202	139	3	9	78	75	53	18	34	10 per cent butter fat in ration.
915	209	146	3	10	51	51	39	23	59	

Keeping in mind that the control Ration 867 (Paper IX) already referred to is a reproduction-deficient diet, we are now ready to examine the effect of addition of butter fat to such a dietary régime, in the absence and presence of cod liver oil (administered separately from the ration). Table VI gives a general summary of the reproduction data of such experiments. When all the fat-soluble vitamins in the diet, containing large amounts of defatted wheat embryo supplying an abundance of vitamin B, were fur-

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nished by 5 per cent butter fat, out of five females one was sterile, three gave birth to first litters only, and another had 3 litters and reared five out of six of the third litter. Lactation was practically a complete failure. When the ration contained 5 per cent butter fat and the animals received in addition fat-soluble vitamins A and D furnished by 0.3 cc. of cod liver oil separately from the ration, continuous fertility was much more assured, although no pronounced difference in degree of success in rearing of young is apparent. When, however, all fat-soluble vitamins are supplied by

TABLE VII.

*Effect of Various Vegetable Oils on Fertility and Lactation.*

0.3 cc. of cod liver oil per animal per day.

Ration No.	Kind of oil.	Oil. per cent	Duration of experiment. days	Reproduction period. days	No. of females.	No. of litters.	Young born.	Young born alive.	Young allowed to be reared.	Young weaned.	
											per cent
938	Wheat.	1.0	175	135	3	7	59	59	36	35	97
940	"	1.0	161	112	3	6	57	57	35	35	100
919	Cocoa butter.	5.0	196	120	3	4	30	30	24	6	25
916	Cottonseed.	5.0	196	114	3	6	53	53	36	18	50
917	Crude corn.	5.0	196	124	3	6*	46*	46	32	25	78
918	Peanut†	5.0	132	40	3	0					

\* Two females are now rearing their third litters and these young are not included in this column.

† Acetone extract of peanuts.

10 per cent butter fat, even in the absence of cod liver oil, not only is continuous fertility absolutely assured, but lactation is also considerably improved; in other words, the addition of 10 per cent butter fat to a synthetic reproduction-deficient diet brings about continuous fertility and notable but not entire success in lactation.

In the light of these data, butter fat cannot be considered as deficient in vitamin E as Evans and coworkers assert.

Tables VI and VII show further evidence of the lactation-promoting properties of vitamin E. A diet, containing only 1 per cent wheat oil, supplemented by 0.3 cc. of cod liver oil per

animal per day, renders a reproduction-deficient diet not only adequate for continuous fertility but also excellent for lactation; while the very same diet containing 5 per cent butter fat, supplemented by equivalent amounts of cod liver oil, though allowing continuous fertility, is followed by considerable infant mortality. The 1 per cent wheat oil Rations 938 and 940 are far superior to even the 10 per cent butter fat Rations 915 and 932. From such statistical data one would naturally infer that vitamin E as furnished by wheat oil is more than an antisterility vitamin, and in addition possesses galactagogic functions. Whether or not there exists a specific fat-soluble galactagogic vitamin we hope will become more apparent from detailed studies now in progress, in which different technique is being employed by taking account of depletion of storage of fat-soluble vitamins during the nursing period.

That cottonseed oil and crude corn oil furnish the antisterility factor when incorporated to the extent of 5 per cent in the diet is also evident (Table VII). The results secured on infant mortality need some comment. Several young of the litters whose mothers partook of rations containing these oils were observed to die with preceding symptoms of beriberi on rations containing an abundance of vitamin B, and it remains yet to be demonstrated what factors in the diet may interfere with the utilization of vitamin B.

In connection with the butter fat problem we are continuing our investigations further. At this writing, excellent results in lactation of first litters are being obtained on Sherman and MacLeod's (6) simple stock ration of whole wheat 66.7, whole milk powder 32.0, and NaCl 1.3. Such a ration contains about 9.0 per cent milk fat. We are extending the observations of Sherman and MacLeod by using skimmed milk powder, instead of whole milk powder, in the presence of not only graduated amounts of butter fat, but also graduated amounts of cod liver oil, with and without graduated amounts of low concentrations of wheat oil. Our observations are including not only records of continuous fertility, but in some cases, also of future generations. The results of such experiments we hope will throw further light on the fat-soluble vitamin requirements for lactation as well as fertility.

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### SUMMARY.

1. The criterion for potency of the antisterility factor must be continuous fertility.

2. Butter fat does not approximate the potency of wheat oil as a source of vitamin E.

3. When butter fat is introduced to the extent of 10 per cent in a synthetic reproduction-deficient diet, not only is continuous fertility assured, but notable success in lactation is at the same time produced.

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## TOXICITY OF ZINC.

BY V. G. HELLER AND A. D. BURKE.

*(From the Chemistry and Dairy Departments, Oklahoma Agricultural Experiment Station, Stillwater.)*

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During the course of an investigation undertaken by this station about 3 years ago in regard to the possibility of poisoning from buttermilk which had been stored in zinc-lined containers, we were impressed, first, by what seemed to be the contradictory findings concerning the toxicity of zinc; second, by the more general distribution of zinc in animal and plant tissue than commonly is assumed by those who have not investigated the subject; and third, by the inadequacy of methods for separating and estimating quantitatively this element when it appears in traces in organic matter.

Since starting this study a series of articles by the coworkers of the Department of Physiology of Harvard School of Public Health has gone far to clear up much of the uncertainty pertaining to certain branches of the problem. The excellent bibliographies of Lutz (1) and of Drinker and Collier (2) call one's attention to the wide distribution of zinc throughout all living matter, and even suggest that it may have a favorable function in life processes. The completeness of their references makes it unnecessary to refer further to the historical works, and those interested in this subject are referred directly to the work of these authors.

The investigations of Batchelor, Fehnel, Thompson, and Drinker (3) who have made an extended study of the physical condition of workers long in contact with zinc and its compounds, likewise lead one to conclude that the earlier investigators might have been somewhat misled in drawing their conclusions. Facts repeatedly cited by these recent investigators indicate that formerly lead, arsenic, or cadmium were probably the contaminating elements which were really responsible for many of the pathological conditions reported.

In our first attack on this problem we were confronted with several difficulties which have been eliminated somewhat by these latter reports. First, we were not certain whether the alleged poisonous conditions reported came from the zinc of the containers or from other elements present. In the course of the investigation we were hampered by the lack of methods of analysis for the element in such small amounts as are to be found in the organs of the rat, which was the animal chosen for our work. We were familiar with and were using the methods of Scott (4), Bodansky (5), and Birckner (6). These methods gave satisfactory results where materials were plentiful, but the completion of the work has been greatly simplified by new methods and modifications as suggested by Lutz (7), Fairhall (8), and Thompson (9). Even with the addition of this later series of investigations, certain phases of the problem concerning the probability of zinc contamination and its effect upon the consumer are still untouched, and this report is offered for those who may be confronted with practical questions in regard to such contaminations of foods.

#### EXPERIMENTAL.

In attacking this problem we first made an examination of the containers in which buttermilk had been stored and found that the zinc surfaces showed evidences of having been dissolved. We next turned our attention to the buttermilk that had been stored in these containers and found from the analysis of the ash by Birckner's method that all buttermilk contained some zinc normally, but that it increased with the acidity of the milk, the length of time stored, and newness of the container. Old surfaces were much less susceptible to the attack of the weak organic acids present. In this work buttermilk that had never been in contact with metallic surfaces was obtained. Definite portions were stored in glass containers and like amounts in zinc-lined vessels for varying periods of time. Definite portions of each of these samples were then drawn off and dried in a vacuum oven and finally ashed at black heat in a muffle furnace, extracted with HCl, and reashed after dampening with nitric acid. The extracts were used and the zinc estimated by the turbidity method of Birckner. These determinations have been recently rechecked by

the improved turbidity method suggested by Fairhall. Table I gives the zinc content of a characteristic sample.

These quantities of zinc present in the buttermilk are suggestive of changes taking place. The figures are variable with samples, probably due to acidity produced by temperature, age, and origin of buttermilk.

Due to the fact that metallic surfaces might contain metals other than zinc, it appeared advisable to determine the toxicity by biological methods, using a normal growing ration, and adding definite amounts of zinc to the ration rather than the buttermilk of unknown contamination. Further, it was desired to know whether the acid radical might have some effect upon its pharmacological actions, and for that reason various salts of zinc were used. Rats were chosen as the test animals because of the ease of hand-

TABLE I.  
*Zinc Content of Buttermilk Samples under Several Treatments.*

Sample No.	Container.	Time of storage.	Zinc.
		hrs.	mg. per 1000 cc.
I	Glass.	24	5
II	Old galvanized pail.	24	15
III	" " "	48	25
IV	New zinc container.	48	33

ling, permitting the observation of large numbers under all kinds of conditions, and because it was of interest to know not only the temporary conditions but also the effect on reproduction and sterility that might be produced by the accumulation of the metal in the body of the animal. The use of rats permitted the study of several generations in a short period of time.

A basal ration was used, which was known to produce satisfactory results for growth, reproduction, and rearing of young in this laboratory. Vigorous young rats were chosen and placed in cages so that each lot would be comparable so far as possible at the beginning of the experiment. One cage of these animals was used as a control lot and was fed a basal ration, while to the rations of the others were added metallic zinc and the various salts of zinc in such quantities as to contain a like amount of the zinc ion. Buttermilk was added to one set of controls and also to the one



zinc ration to see whether the protein of the milk might combine with the zinc and prevent its absorption, it being known that such proteins are often used as antidotes against certain metallic poisons due to the formation of insoluble compounds. The object in this test was to ascertain whether, due to this combination, milk that was contaminated with zinc was less dangerous than other foods so contaminated.

TABLE II.

Lot No.	Ration.	No. of males.	No. of females.	No. of litters.	Young born.	Young lived.	Young died.	Growth.*
I	Basic (B).	3	1	2	18	16	2	++++
II	B + buttermilk.	2	2	2	11	11	0	+++++
III	B + 0.25 per cent Zn dust.	2	2	6	43	36	7	++++
IV	B + 0.25 per cent Zn as ZnCl <sub>2</sub> .	2	2	4	25	20	5	++++
V	B + 0.25 per cent Zn as ZnCO <sub>3</sub> .	2	2	7	53	50	3	++++
VI	B + 0.25 per cent Zn as ZnCO <sub>3</sub> + buttermilk.	2	2	4	31	30	1	+++++
VII	B + 0.25 per cent Zn as ZnSO <sub>4</sub> .	2	2	7	44	41	3	++++
VIII	B + 0.5 per cent Zn as ZnO.	2	3	2	13	11	2	+++
IX	B + 0.5 per cent Zn as ZnCl <sub>2</sub> .	2	7	7	46	27	19	++++

\*++++ represents normal growth.

The basal ration used in these tests consisted of:

	per cent		per cent
Yellow corn.....	53	NaCl.....	1
Ground whole wheat.....	20	CaCO <sub>3</sub> .....	1
Oat.....	10	Alfalfa meal.....	5
Tankage.....	10		

Table II gives the ration supplements used as well as other information gathered concerning growth and reproduction when these rations are fed.

The results of the findings were unexpected and surprising for the reason that while 0.25 per cent of zinc is not a great amount of a metallic ion in a ration, it is far in excess of any ordinary contamination and an amount such as would prove fatal for a toxic metal. No toxic effect was obtained; the rats grew normally. No noticeable difference could be noted between those eating the basal ration and those receiving the additions of zinc salts. Neither could we observe any great difference in the effect of the anion. Growth was normal, mating took place at the normal age, and young were born and reared in the various cages in comparable manners. The general appearance of the animals in clearness of eye, smooth-

TABLE III.

Weight of.....	Animal.	Heart.	Liver.	Kidneys.	Spleen.	Lungs.	Testicles.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Normal.....	225	0.7856	12.199	0.9513	0.6387	0.999	2.5126
0.25 per cent ZnCO <sub>3</sub> .....	230	0.7853	10.987	1.1632	0.6989	1.0661	2.9973

TABLE IV.

	Weight of animal.	Ash of:					
		Heart.	Liver.	Kidneys.	Spleen.	Lungs.	Testicles.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Normal.....	0.225	0.0081	0.0808	0.0247	0.0300	0.0179	0.0156
ZnCO <sub>3</sub> .....	0.230	0.0113	0.0831	0.0285	0.0245	0.0189	0.0149

ness of coat, and ease of movement did not indicate any deleterious effect. The offspring were continued on similar rations, and the third generation still maintains the same vigor in growth and reproduction as the animals receiving the growing rations. It was observed that the addition of buttermilk *ad libitum* did increase the vigor of both the lots receiving the basic ration as well as those containing zinc. The addition of milk to all growing rations, however, usually results in such accelerations.

After full growth had been reached, an autopsy was made and the various organs examined. So far as could be observed no lesions or other pathological conditions were evident. The organs were removed, carefully washed free of blood, measured,

weighed, and compared to the organs of normal animals. Table III presents the average findings of ten or more animals in each case.

The organs were next ashed at black heat in the muffle furnace and the ash content determined. Table IV presents the average ash content of organs in each case.

TABLE V.

Organ used.	Average weight of organ.	Average weight of zinc found.	Average concentration of zinc.
	<i>gm.</i>	<i>mg.</i>	<i>mg. per gm.</i>
Heart.			
Normal.....	0.7856	0.011	0.014
Zinc-fed.....	0.7853	0.014	0.018
Lungs.			
Normal.....	0.999	0.02	0.021
Zinc-fed.....	1.066	0.024	0.023
Liver.			
Normal.....	12.199	0.2439	0.02
Zinc-fed.....	10.987	0.2858	0.026
Spleen.			
Normal.....	0.6387	0.0198	0.031
Zinc-fed.....	0.6989	0.0265	0.038
Kidneys.			
Normal.....	0.9513	0.0143	0.015
Zinc-fed.....	1.1632	0.0163	0.014
Testicles.			
Normal.....	2.5126	0.032	0.013
Zinc-fed.....	2.9973	0.051	0.017

Another set of organs was ashed at black heat and the ash was extracted with 1:1 redistilled HCl, and analyzed for its zinc content by the micro method of Lutz (7) which is briefly outlined as follows: The HCl extract was treated with cupferron to precipitate the iron and copper. The filtrate is treated with sodium acetate until the mineral acids are neutralized as indicated by methyl orange. 5 cc. of dilute copper nitrate solution were added

to serve as an entrainer and hydrogen sulfide added which precipitated the zinc together with the copper. The precipitate was washed with water and hot alcohol to free it of foreign matter. This residue was dissolved in hot nitric acid and evaporated to dryness, redissolved in hydrochloric acid, and the copper again precipitated with hydrogen sulfide. The filtrate containing the zinc was evaporated just to dryness and taken up in water. Aliquots of this solution were added to Nesslerizing tubes containing alcohol. Urobilin was added, and the fluorescence obtained compared to zinc standards simultaneously prepared in a similar manner. Table V gives the zinc content of organs of normal and zinc-fed animals.

Since no noticeable deleterious effects of the zinc feeding were found through three generations of observed animals and chemical tests failed to reveal much accumulation in the organs of the

TABLE VI.

Food eaten.	No. of animals.	Time of observation.	Weight of dry feces.	Zinc.			
				Consumed.	In feces.	In urine.	Stored and unaccounted for.
gm.		hrs.	gm.	mg.	mg.	mg.	mg.
70	3	36	21	525.7	494	10	21.7

animals, we next turned our attention to the path of elimination of the zinc from the body. In order to gather the urine and feces uncontaminated, it was first necessary to build a metabolism cage free from all metals. This was accomplished by building upon a large shallow porcelain dish two perforated discs, the upper to serve as a floor for the cage and the lower to collect the feces but to permit the passage of the urine. These floors were made of small glass rods set in concrete rings cast so as to fit exactly the dish but still be removable. The concrete was treated to be non-porous. The sides of the cage were also made of glass tubes set in concrete rings above and below. A deep glass container protected with an upper flange was suspended in the cage permitting the rat to secure food without wasting any or contaminating the urine or feces, as well as making it possible to measure exactly the amount of food consumed. A drip tube

furnished distilled water for drinking purposes. The rats were kept in this cage over long periods of time to accustom them to their surroundings. When ready for study the amount of food eaten, the weight of the dry feces, and the weight of urine residue were noted over definite periods of time as recorded in Table VI. The figures are the average of five determinations from rats 6 months of age. The zinc was fed as zinc oxide added to the basal ration.

The samples of feed, feces, and urine were ashed, extracted, and estimated for their zinc content by the modified turbidity method suggested by Fairhall (8).

An examination of these figures indicates that zinc if absorbed, is again eliminated from the body primarily with the feces. Large storage in the internal organs was in no case evident. The urine, while showing an increased content, does not appear to furnish as important a path of excretion as do the feces. A similar observation was recently recorded by Drinker, Fehnel, and Marsh (10) in their study of the elimination of zinc in the feces of a normal man.

The feeding of commercial samples of buttermilk and zinc both to the rat and to larger animals is being continued in cooperation with members of the Dairy Department and will be reported at a later date.

#### SUMMARY AND CONCLUSIONS.

1. Buttermilk normally contains a small amount of zinc, the amount increasing after contact with zinc containers, and varying according to the duration of contact, acidity of the buttermilk, and the newness of the zinc surface exposed.

2. Zinc added to a normal ration either in the form of pure zinc dust, zinc oxide, or certain zinc salts, in amounts as great as ever found in contaminated foods did not interfere with growth, reproduction, and normal functions of the rat through three generations.

3. No pathological conditions were found in the organs of rats fed the rations used in this experiment.

4. The total ash content of the organs studied showed no perceptible increase.

5. Zinc is found normally in the internal organs of rats fed growing rations. Only small increase was observed in the organs of zinc-fed animals.

6. The path of excretion is primarily through the feces, though the content in the urine is slightly increased in the zinc-fed animals.

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# THE ASSOCIATION OF VITAMIN A WITH GREENNESS IN PLANT TISSUE.

## I. THE RELATIVE VITAMIN A CONTENT OF HEAD AND LEAF LETTUCE.\*

BY MARIE DYE, OLIN C. MEDLOCK, AND JOHN W. CRIST.

(From the Division of Home Economics† and the Department of Horticulture, Michigan State College, East Lansing.)

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### INTRODUCTION.

The question of whether vitamin A is associated with the pigment of plants is of considerable importance in horticulture since many horticultural products are marketed after being bleached or blanched to different degrees by either natural or artificial processes. The reason for doing this is to improve the quality as determined by appearance, tenderness, and palatability. If, however, the amount of vitamin A in the plant is materially reduced by rendering it less green or even non-green, thus decreasing its value as a food, it would seem that our present standards of quality are somewhat superficial.

The experiments to be described in this paper were designed to study the comparative vitamin A content of head and leaf lettuce, of the leaves of lettuce from the inside and outside of the head, and leaf lettuce grown in the hothouse and also out of doors.

### LITERATURE.

Steenbock and Sell (1) found that the green, inside leaves of cabbages which had failed to head properly, contained 10 times as much pigment as white leaves on the inside of good heads and were far richer in vitamin A, although some growth occurred in the rats fed on the white leaves. Later

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Steenbock, Sell, and Boutwell (2) used ripe peas with green color in various intensities and found that out of six samples investigated, those of a green color, *also carrying considerable yellow pigment*, were far richer in their vitamin A content than yellow peas which contained much less yellow pigment. They stressed the differences in yellow pigment rather than the equally or even more striking differences in green pigments. In 1920 Osborne and Mendel (3) dried the foliage of spinach, young clover, alfalfa, and grass plants and made an extraction with ether. The residues remaining from the extracts promoted recovery and renewal of growth in rats declining in weight on diets deficient in this vitamin.

Early results obtained by Coward and Drummond (4) when working with white cabbage leaves, etiolated seedlings, and certain algae and fungi led them to the conclusion that the amount of vitamin A does not increase in germination but only in those plant tissues in which photosynthesis is going on. Since chlorophyll is essential to photosynthesis, this would signify that the increase occurs only in tissues that become green. Later Coward (5), besides reconfirming these conclusions regarding the inferiority of etiolated seedlings (fed fresh) as compared with green, reviewed and explained Wilson's (6) results that were contrary to her own. She, also, found that though light is necessary to the formation of vitamin A in plant tissues, the process is independent of carbon dioxide, and oxygen in the surrounding air, of the ultra-violet rays of the spectrum, of calcium salts in the culture medium, and finally of the presence of chlorophyll in the plant. These results, contrary to earlier ones, seem to indicate that the formation of vitamin A is independent of photosynthesis. However, these results can hardly be taken as fully conclusive since no duplicate tests were made and the number of rats used for each experiment was small. Coward's method for obtaining atmospheres, free from carbon dioxide or oxygen, is open to question and seedlings grown in such an atmosphere are probably very abnormal whether etiolated or green. It would seem that tests based on the use of plants grown in the dark and in the light, both lots having normal atmospheres, would furnish results more reliable and comparable. Widmark (7) on the other hand, has shown that whenever plants lose the ability to form chlorophyll or lipochrome, they also lose the ability to form vitamin A even if kept in full illumination. Clearly the problem has not been solved and our results do not settle the matter but add to the weight of evidence supporting the theory of the association of vitamin A and greenness in plants.

#### EXPERIMENTAL.

The rats used in these experiments were from our own stock and were of known pedigree. They were selected from litters of five to ten each, when they were from 28 to 30 days of age and weighed between 30 and 50 gm. each. They were placed on a basal diet of corn-starch 78 per cent, purified casein 18 per cent,

and salt mixture (McCollum's No. 185<sup>1</sup>) 4 per cent. Each animal was given daily 0.5 gm. of dried Fleischmann yeast. The corn-starch was irradiated for 15 minutes at a distance of 14 inches by a Cooper Hewitt quartz mercury vapor lamp run with a voltage of 110 at 4.5 amperes. Steenbock (8) and Dutcher (9) have each shown that animals on this type of ration grow better if either the animals or the food is irradiated. The former found that irradiated corn-starch would prevent the development of rickets and the latter that irradiated dextrin fed at a level of 76 per cent gave a higher per cent of ash in the bones of animals fed on a low vitamin A diet. We have found that animals grow for a longer time and are in better physical condition when the corn-starch is irradiated. The casein, obtained at the College dairy, was purified by the method of Sherman and Kramer (10).

The animals from a litter were distributed uniformly as far as possible in the different experiments and one left on the basal ration to serve as a negative control. Approximately ten animals were used for each lot in each experiment. The feeding of the lettuce began when the animals had reached the point of constancy in weight or had begun losing weight, and continued for 8 weeks. When head lettuce has matured, the interior leaves are bleached to a yellowish color and only a few of the outside leaves remain conspicuously green, while those between show gradual variations of greenness. In these experiments either the green outside leaves or the inner bleached ones were used. In both the head and leaf lettuce the outer edge of the leaf was fed. Since it was impossible to grow lettuce that was well headed, market head lettuce was used. The leaf lettuce was grown on the College farm or in the hothouses.

### Results.

*Experiment 1.*—In this experiment twelve animals were fed on the yellow inside leaves of head lettuce, thirteen on leaf lettuce, and eight had no vitamin A supply, serving as negative controls. 0.3 gm. of fresh lettuce was fed daily, this amount having been indicated by preliminary experiments to be a quantity well suited for the production of a desirable rate of growth. The results are shown graphically in the unbroken curves of Figs. 1 and 2. The curves are plotted cumulatively from weekly averages.

<sup>1</sup>McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

The animals recovered and resumed growth promptly when the feeding of lettuce was started. The graphs show clearly the superiority of the leaf over the head lettuce as a source of vitamin A. The total average gain for a period of 8 weeks of the rats on leaf lettuce was 64 gm. and of those on head lettuce was 34.2 gm.

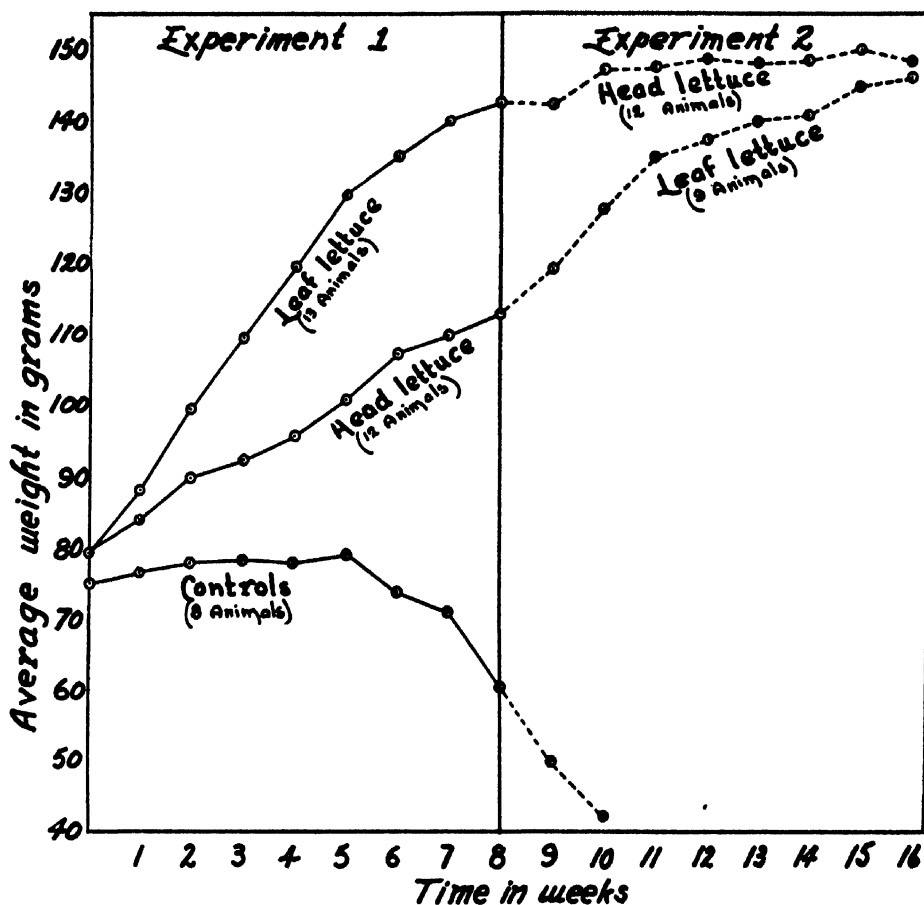


FIG. 1. Growth curves for animals of Experiments 1 and 2.

The second set of curves (Fig. 2) is very interesting. Average gains per unit of weight are plotted against time in weeks. Naturally, a certain amount of the vitamin is required to support the organism itself, this amount being dependent upon the size of the animal. Any surplus is available for the facilitation of growth or increase in size. The wide separation of the two curves emphasizes the general fact of a far greater surplus of vitamin A in

the 0.3 gm. of leaf lettuce than in the same quantity of head lettuce. Maximum rate of growth for rats on leaf lettuce was not realized until the end of the 2nd week, while this point appeared at the close of the 1st week with the other group. The two graphs cross at a point—abscissa 5.5, ordinate 64—and tend downward together thereafter, both approaching the state of zero gain which obtained at the beginning. It should be realized that the animals on leaf lettuce are larger than the others and that where the curves cross the average size of these is 29 per cent greater than for the average rat on head lettuce.

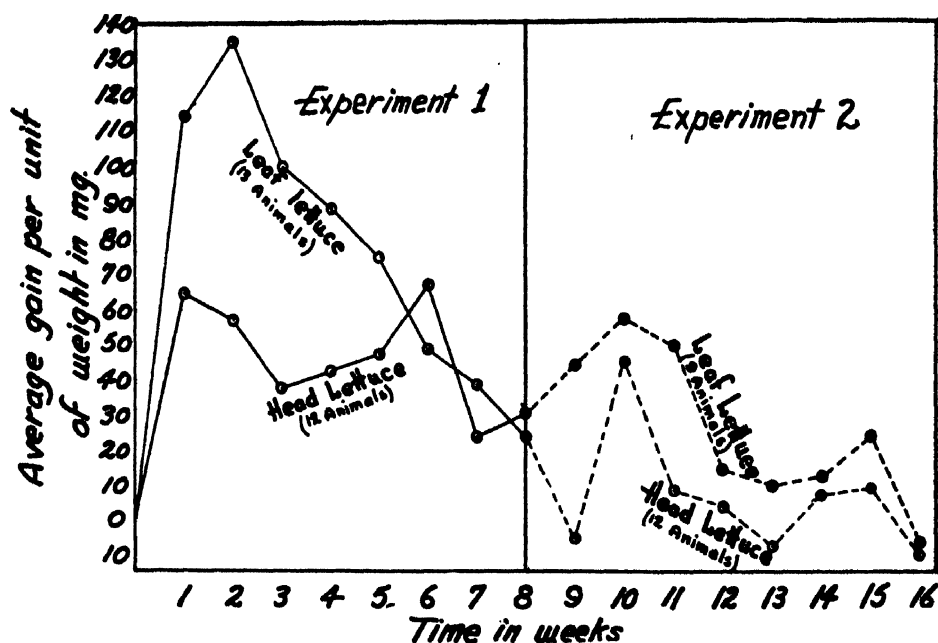


FIG. 2. Growth curves for animals of Experiments 1 and 2.

*Experiment 2.*—At the end of 8 weeks the lettuce diet of the two groups of rats was reversed, so that the one which had been receiving leaf lettuce was changed to head lettuce and *vice versa*. The rest of the diet and the manner of feeding remained the same.

This plan was adopted in hope of minimizing the effect of any variation due to either heredity or peculiarities of physiology as reflected in behavior of the animals with respect to eating and drinking. Variation is considerable at best, especially with those rats on the diet more deficient in the vitamin. They are not in

the best biological tone, it seems, and hence tend to eat (except the fresh foliage) and drink more indifferently and spasmodically. The number of animals per lot necessary to secure mathematically significant weekly averages of gain would be impossibly large. Reversing the fresh foliage diet of the same lots of rats serves to reduce the bearing of variation on the results by making each animal or each lot of animals its own check. The results are shown in Figs. 1 and 2, the broken curves representing the growth following the reversal of the lettuce diet of the first 8 weeks.

The animals which were switched from leaf to head lettuce made no appreciable gain for the ensuing 8 weeks, but did hold at approximately constant weight. This may indicate that there was a storage of vitamin from the leaf lettuce of the previous 8 weeks, which amount together with what they obtained from the head lettuce was sufficient to sustain the organism at a constant level of weight. Sherman and Kramer (10) have shown that the body of the rat can store vitamin A and that when the animals are put on a diet devoid of vitamin A, the vitamin deficiency of this experimental diet and the vitamin content of the preceding diet together determined the length of the survival period. On the other hand, the animals switched from head to leaf lettuce began at once to gain rapidly and continued to gain until the end of the experiment when they had reached a weight practically identical with that of the other lot. The steepest part of the entire curve for the 16 weeks is between the 8th and 11th weeks, or the 3 weeks following the change.

*Experiment 3.*—To determine whether the differences just recorded were associated with greenness or were more or less inherent in the type of plants, a comparison was made of the effect of feeding the inner leaves and the outside leaves of the same heads of lettuce. The outside leaves which are left after the trimming of the head for market are distinctly green although less green than the leaves of leaf lettuce, while the innermost leaves are decidedly yellow and contain but little if any chlorophyl. The basic diet was the same as in the two previous experiments. The amount of lettuce of each type fed daily was 0.2 gm. instead of 0.3 as this quantity had been shown to have produced a slower and more satisfactory rate of growth.

The results, given graphically in Figs. 3 and 4 show that the

animals fed on the inside yellow leaves of the head lettuce made no gains of any consequence but did maintain approximately the average weight possessed in the beginning, except for two of the number that died before the end of the period. Xerophthalmia was prevalent among them throughout the 8 weeks. The lack of any considerable loss in weight could be taken to indicate the presence of at least a small proportional quantity of vitamin A in the plant tissue provided in their diet.

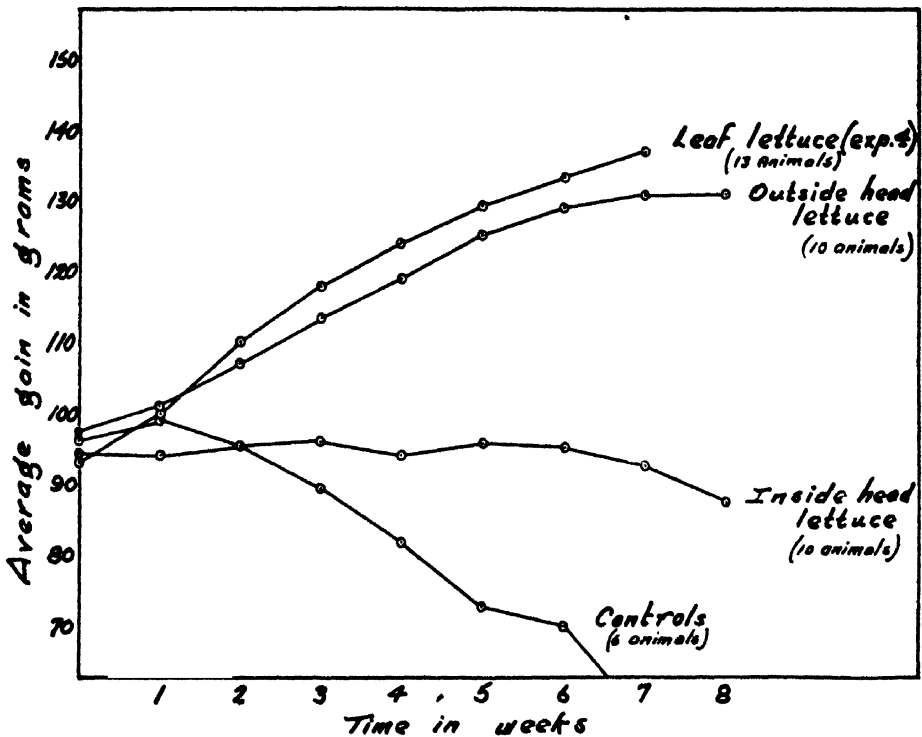


FIG. 3. Growth curves for animals of Experiment 3.

In contrast to this lot of rats, those fed on the same quantity of the greenest outside leaves of the head lettuce made large and consistent gains and were free from disease. Since Experiments 3 and 4 were under way at the same time, the curve for the animals on greenhouse leaf lettuce in Experiment 4 is produced with the curves of Experiment 3. It can be noted that these animals made more rapid and greater gains even than those fed on the outside leaves of head lettuce. The growth curves derived by plotting gains per unit of weight against time serve to emphasize

doubly the superiority of the green outside leaves of the head lettuce.

*Experiment 4.*—Since ordinary greenhouse glass filters out a portion of the ultra-violet rays of the sunlight spectrum, this experiment was conducted as a test of the vitamin A content of glass house leaf lettuce and leaf lettuce of the same variety grown in the open. Details of the work were the same as for Experiment 3 except that the experiment covered only 7 weeks. 0.2

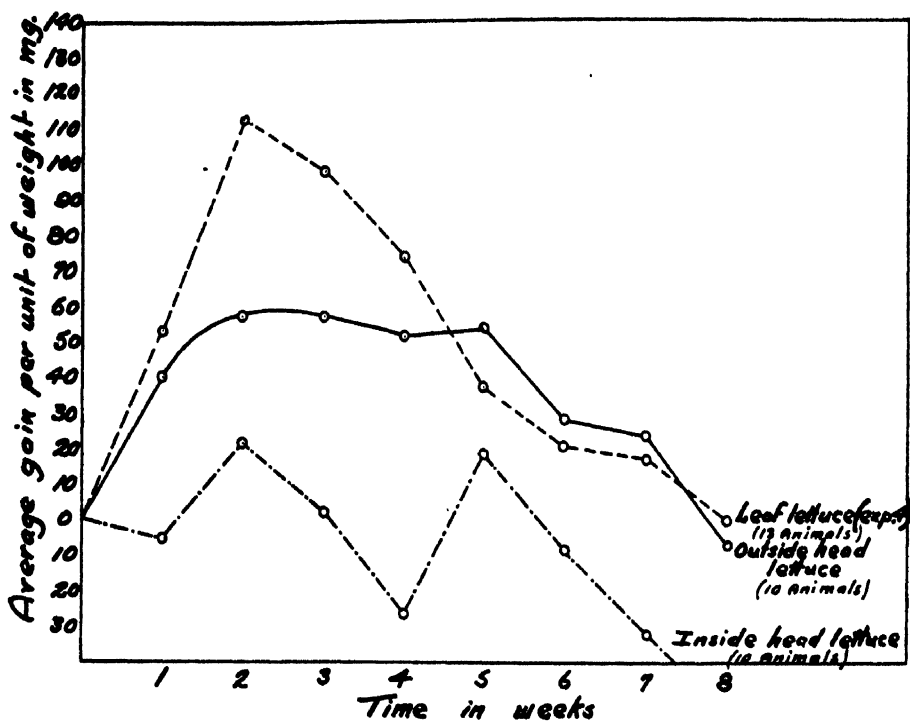


FIG. 4. Growth curves for animals of Experiment 3.

gm. of lettuce was fed daily. The data are presented in Figs. 5 and 6.

The curves plotted from the average weights indicate, considering that the animals on out of door lettuce averaged 3 gm. heavier at the beginning than the others, that the growth in the two lots was almost identical. The graphs, based on average gains per unit of weight, give some indication of superiority in the glass house lettuce after the 1st week until the 5th week, with the out of door grown lettuce slightly better before and after

these dates, though the differences are probably insignificant. It should be noted that the work was done during the autumn months

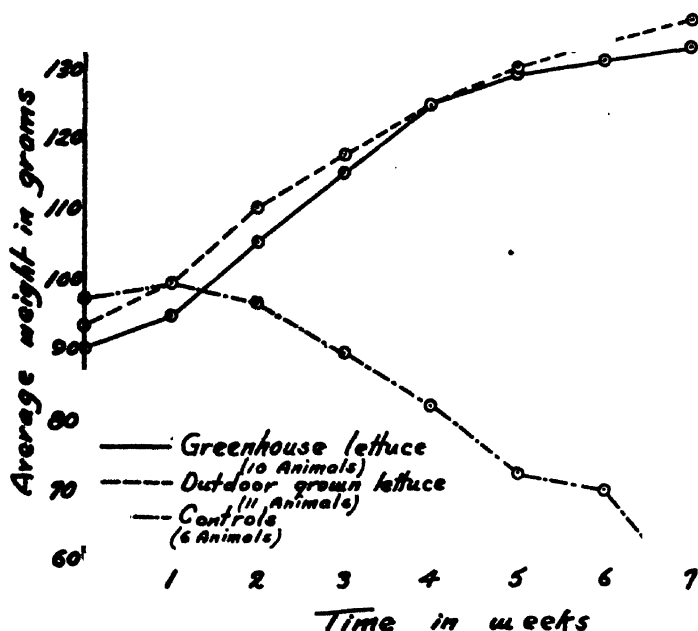


FIG. 5. Growth curves for animals of Experiment 4.

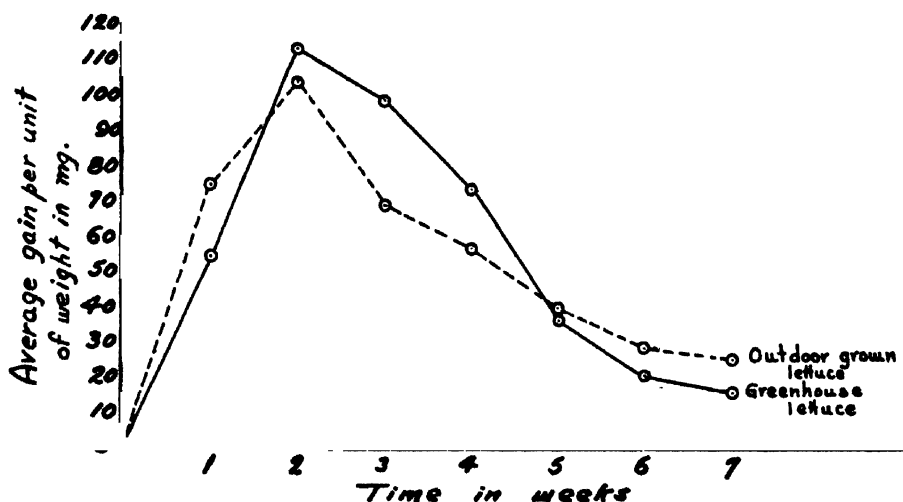


FIG. 6. Growth curves for animals of Experiment 4.

and the lettuce grown outside was exposed to lower temperatures, especially towards the close of the experiment. What differences this might have made one way or the other is not known.



## DISCUSSION.

The evidence which has been submitted emphasizes the possibility of some connection between the presence of chlorophyll or greenness in the lettuce leaf and the vitamin A content. However, it would be unsafe to infer positively either a causal relationship or an indissoluble association of the two things from the total amount of available data. Perhaps the most serious obstacle is the exception shown by the fact that white leaves and even yellow leaves of such plant parts as cabbage and lettuce heads and the foliage of etiolated seedlings afford the vitamin to a slight extent. The following considerations may serve in harmonizing this apparent exception with the theory.

It can hardly be affirmed that all the leaves which appear more whitish than green in the mature head of lettuce or cabbage have ever been wholly non-insolated. While the head is loose, at least some light must reach the leaves directly and some by transmission. They are still somewhat green in the mature head and may have been still greener before the growth of the innermost leaves made the head become fully compact. Would their vitamin A content disappear entirely as a consequence? Would their chlorophyll disappear altogether or merely be reduced to a lower rudimentary form? Coward's (5) work with *Helianthus* seedlings showed that a store of the vitamin once formed is not immediately used up by the plant.

The investigations of Monteverde (11) and Lubimenko (11) led them to state that a pigment called "chlorophyllogen" is formed independently of light in the chromatophores of all potentially green plants. This pigment, or mother substance of chlorophyll is transformed into chlorophyll at once under the influence of light, but in some species (ferns, evergreen seedlings (*Larix*, *Pinus*, *Picea*)) it becomes chlorophyll in darkness. Issatchenko (11) and Liro (11) showed that its transformation into chlorophyll took place in the absence of oxygen and carbohydrates and even under unfavorable temperature conditions. In the June and July numbers of the *Revue générale de botanique* for 1926, Lubimenko (12) in summarizing his extensive investigations and also the present situation regarding the pigments of the plastids and photosynthesis, strongly reaffirms his position concerning chlorophyllogen. Most embryos develop a green color

during the first stages of their development after fertilization. In twelve of the 110 families of Dicots, Monocots, and Gymnosperms examined spectroscopically by him, green color was present in the embryos even after the full maturity of the seeds had been accomplished. In the mature seeds of the cucurbits a rich store of chlorophyllogen is found in what were the inner integuments of the ovule. Lubimenko states that the seedlings of Angiosperms produced in the dark accumulate the yellow pigments and a very small quantity of a green pigment called chlorophyllogen. As a general rule, the quantity of chlorophyllogen in the seedlings is so small in proportion to the quantity of yellow pigments, that this pigment has no sensible influence on the color of the seedlings. With the Angiosperms, the chlorophyl is replaced in the dark by the chlorophyllogen of which the accumulation, as in the case of chlorophyl, depends primarily on the organic nutrition of the plastids. The summation of his evidence is the conclusion that the development of the plastids themselves, as well as the development of the various pigments in the plastids, is more dependent on the organic nutrition of the cells than any other factor, light included. In view of these considerations it may be possible that in apparently chlorophyllless structures, there is a close approach to chlorophyl, indeed, the existence of certain leuco- and chlorophases of chlorophyl which could be related to vitamin A formation in a significant manner.

#### SUMMARY.

1. Leaf lettuce exceeded head lettuce in the promotion of growth in rats that had ceased to gain on a diet deficient in vitamin A.
2. The outside, green leaves of head lettuce were far superior to the inside yellow leaves in furnishing vitamin A.
3. Indoor leaf lettuce proved as beneficial as outdoor leaf lettuce in producing growth.
4. Though no certain identity of chlorophyl or any of its primary phases with vitamin A in lettuce tissue has been proved, the evidence points plainly towards the probability of some close relationship between the two.

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# A QUANTITATIVE STUDY OF THE DETERMINATION OF VITAMIN B.\*

BY H. C. SHERMAN AND E. H. MACARTHUR.

(From the Department of Chemistry, Columbia University, New York.)

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Pending the isolation of vitamins as pure substances, quantitative determinations of vitamin values (or relative vitamin contents) of plant and animal materials are being made by comparing the results of quantitatively conducted feeding experiments and expressing the value of each material as tested either by comparison with some previously tested material or in terms of the response obtained in a standard test animal. Of these two ways of expressing results the latter seems preferable, and is rapidly coming into use. This accentuates the importance of quantitative work upon the various factors which may influence the response of test animals to their vitamin intake.

The present paper presents briefly the results of such an investigation of the quantitative determination of vitamin B by means of growth experiments with young rats.

## EXPERIMENTAL.

### *Procedure.*

Healthy and normally growing white rats of 28 to 29 days of age, from families of known breeding and nutritional history, are placed in individual all metal cages with raised wire screen floors to prevent access to excreta, and are fed during an experimental period of 8 weeks with predetermined graduated allowances of the material to be tested as source of vitamin B, while furnished *ad libitum* with a basal diet adequate for all other

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nutritional needs of the rat but devoid of vitamin B. The material under test thus becomes the sole source of the one vitamin for which it is being tested, and the concentration of this vitamin in the material may be judged to be inversely proportional to the amount of material which need be fed, if the test animal and all the conditions of the test are quantitatively standardized and controlled. Each animal is weighed at least once each week, and the amount of food consumed during each weekly period is recorded.

The *basal diet* used by us as the result of an extended previous investigation in this laboratory<sup>1</sup> consists of: purified casein, 18; butter fat, 8; cod liver oil, 2; Osborne and Mendel salt mixture, 4; starch, 68 per cent.

The casein was freed from vitamin B by extraction in the cold with 60 per cent (by weight) alcohol as follows: Each 200 gm. of casein was mixed for  $\frac{1}{2}$  hour with 1 liter of the alcohol by means of a mechanical stirrer and then allowed to stand for  $5\frac{1}{2}$  hours, filtered on a Buchner funnel, and washed with 500 cc. of 60 per cent alcohol. The casein was again treated with 1 liter of 60 per cent alcohol and allowed to stand 18 hours, then filtered, and washed as before with 500 cc. of 60 per cent alcohol and finally with 500 cc. of 90 per cent (by weight) alcohol to facilitate subsequent air-drying.

*Quantitative Relations of Gains in Body Weights of Experimental Animals to Relative Amounts of Vitamin B Fed.*

The average results of feeding different graded allowances of vitamin B are summarized in the weight curves of Series I in Fig. 1, which show the average gains of four lots of ten rats each, the lots being well matched as to family origin, sex, and initial size, and receiving respectively 3, 4, 5, and 6 cc. of a uniform (partially) evaporated milk per capita six times per week as sole source of vitamin B. On the lower allowances, some of the animals died during the experimental period, so that the latter portions of the curves designated as 3 cc. and 4 cc. (Series I of Fig. 1) represent the average records of the survivors—which were doubtless somewhat more vigorous than the average of all.

<sup>1</sup> Sherman, H. C., and Spohn, A., *J. Am. Chem. Soc.*, 1923, xlv, 2719.

In order that practically all of the animals submitted to experiment may be represented in the final average result, while at the same time the growth is so restricted that any increased intake of the vitamin will certainly be reflected in a greater gain in weight, it seems probable that a rate of gain such as that resulting from the feedings of 5 cc. in the experiments represented by Fig. 1, represents approximately the best level at which to make quantitative comparisons. A large number of additional experiments was therefore made at this level of vitamin B intake with the average result shown in Series II of Fig. 1. It will be noticed that

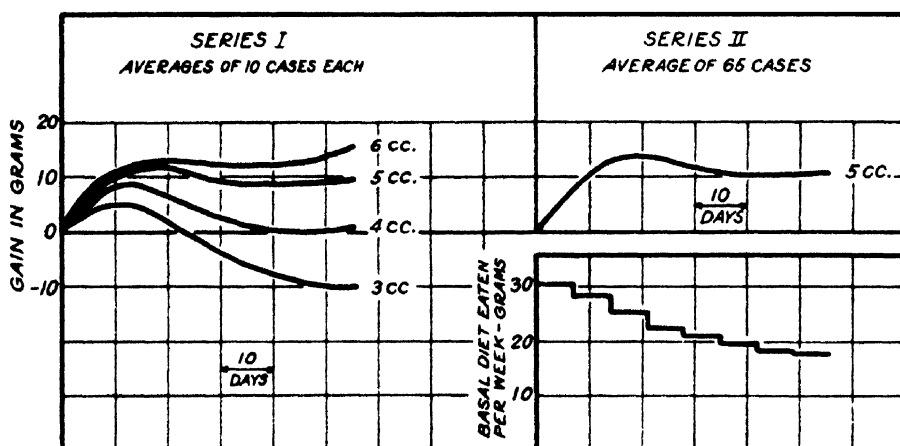


FIG. 1. Series I, curves showing the average gains in weight, of ten directly comparable cases each, made by rats fed varying dosages of evaporated milk as sole source of vitamin B. Series II. Above, a composite curve of 65 cases, showing average gains made by rats receiving the dosage of vitamin B which is probably best adapted to its quantitative measurement. Below, a chart showing the average gm. of basal diet eaten per week by the 65 rats.

with the intake of vitamin B restricted to the level here proposed as best suited to quantitative comparisons (and with a basal diet excellently adapted to all other nutritional needs), there is first a distinct gain in weight for 2 to 3 weeks, then a slight decline for a similar period, followed by a considerable period during which the weight remains approximately constant. Naturally, such smoothness of the weight curve is only to be expected in averages of experiments sufficiently numerous to minimize the effects of individual variability. The experimenter must also be prepared

to meet occasional differences in the response of the weight curve to graded allowances of the vitamin according to the nature of the material fed, which may affect the animal's appetite or capacity

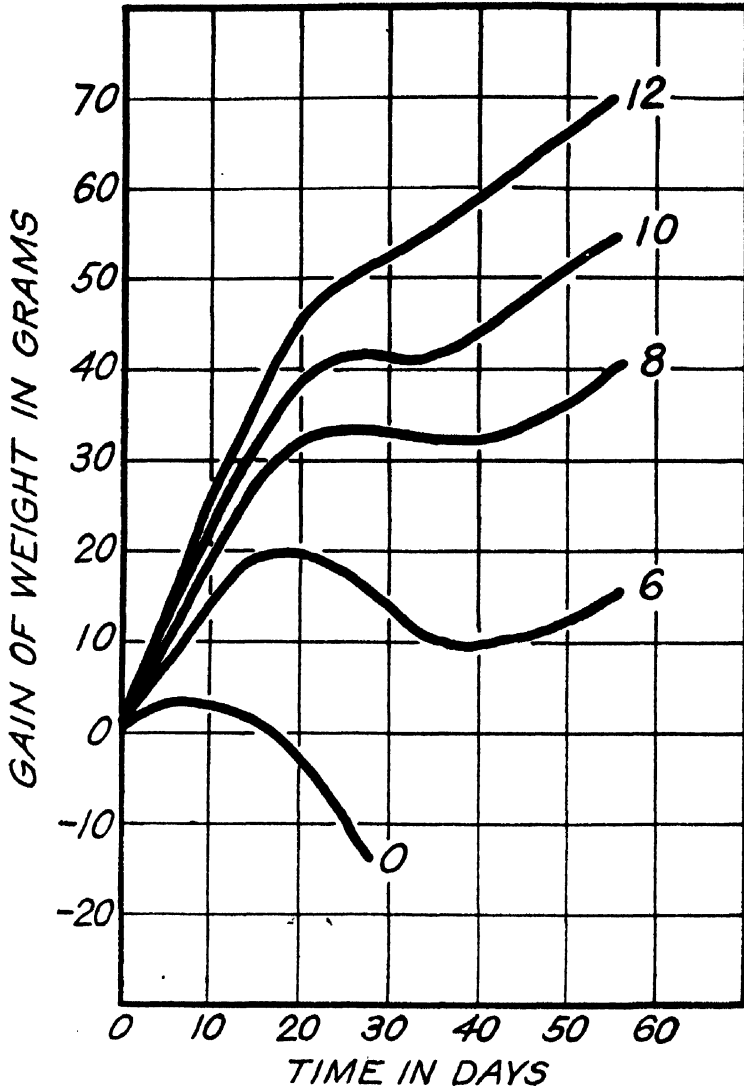


FIG. 2. Weight curves resulting from different intakes of vitamin B in the form of skimmed milk powder (experiments of Sherman and Spohn).

for the basal diet. Figs. 2 and 3 represent differences in (weight curve) response to largely increased dosages of foods of different type, and serve further to emphasize the desirability of working

at relatively low levels in order to minimize differences due to such causes, and to give to the estimates of relative amounts of the vitamin in different materials as high a degree of quantitative accuracy as is possible. For the smaller the amount of material that need be fed to test its vitamin value, the less is the danger that its other constituents or its physical properties may have an appreciable influence upon the results of the experiment.

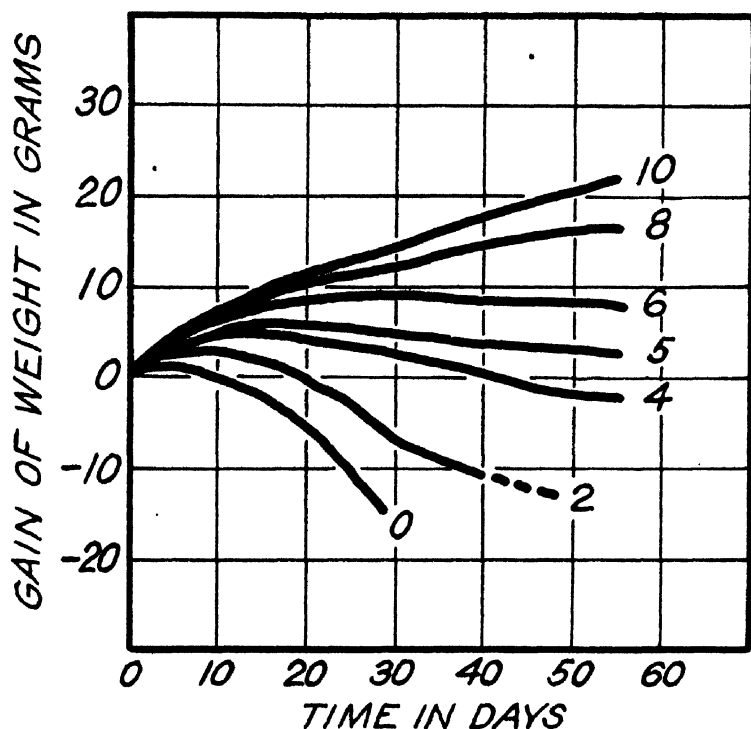


FIG. 3. Weight curves resulting from different intakes of vitamin B in the form of tomato juice (experiments of Sherman and Grose).

#### *Length of Experimental Period.*

The weekly weighings of 65 experimental animals fed at the level of vitamin B intake recommended above, have been averaged and the coefficient of variation and probable error of average weight for each weekly weighing have been computed. There is no significant difference in the probable errors of the means or in the variability of the results such as would in itself serve as a guide to the best length of experimental period; but the general



bearing of the data of these and other experiments and the typical form of the weight curve as explained above, confirm us in the view that an experimental period of 8 weeks is somewhat preferable to a shorter period, such as 6 weeks.

*Influence of a Preliminary Depletion Period.*

Steenbock, Sell, and Jones<sup>2</sup> found no evidence of any significant ability to store vitamin B in young rats from 3 to 6 weeks of age. Osborne and Mendel<sup>3</sup> have, however, definitely shown a difference in the vitamin B content of the livers of animals according as they had been fed a normal diet or one lacking this vitamin; and in the case of vitamin A it is now known to be extremely important that the body of the experimental animal be depleted of its surplus before quantitative feeding experiments are begun. Hence it seemed desirable to test the effect of a preliminary depletion period in experiments for the measurement of relative amounts of vitamin B. Twenty-one careful comparisons were made, in each of which one rat of a litter (from a previous diet of whole wheat and whole milk) was subjected to a preliminary depletion period by being kept for 10 days or until gain in weight had ceased upon the basal diet only, then fed the limited amount of food for the determination of vitamin B, while a twin of the same sex and initial weight was fed without the depletion period. The uniformity of the results was not enhanced nor was any other advantage found to result from the interposition of the depletion period and we therefore conclude that, important as is the depletion period in feeding experiments for vitamin A, it is of no advantage in the case of vitamin B, at least under conditions such as obtain in our investigations.

*Influence of Sex and of Initial Weight upon Results of Feeding a Constant Allowance of Vitamin B.*

Osborne and Mendel<sup>4</sup> found that the amount of vitamin B required is approximately proportional to the size of the experi-

<sup>2</sup> Steenbock, H., Sell, M. T., and Jones, J. H., *J. Biol. Chem.*, 1923, lv, 411.

<sup>3</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1923-24, lviii, 363.

<sup>4</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1922, liv, 739.

mental animal (rats). Their larger animals were also older. If differences in size or in sex among experimental animals of the same age influence their vitamin B requirements, these factors should be taken into account in quantitative work upon vitamin B by the rat-feeding method.

We have therefore taken the records of the animals most directly comparable in all other respects, separated the sexes and divided each into four groups according to initial weight (*i.e.*, body weight at 4 weeks of age when the experimental feeding was begun), and averaged the initial weights and gains in weight with the results shown in Table I.

TABLE I.  
*Comparison of Initial Weights and Average Gains of Males and Females.*

Group No.	No. of cases.	Average initial weight.	Average gain for 8 wks.
Males.			
I	10	34.10	18.80
II	12	44.17	12.08
III	12	54.58	10.25
IV	13	67.38	7.77
Females.			
I	9	36.78	17.11
II	9	50.33	7.00
III	9	53.89	8.33
IV	9	64.44	1.67

In these groups, where, however, the numbers are necessarily small, it will be noticed that the average gain in weight for 8 weeks for the males was greater than that of the females for a corresponding group, and that both among the males and among the females, increasing initial weights have resulted in smaller average gains during the 8 weeks of the experimental period, with uniform per capita allowances of vitamin B.

As a further test, all available cases regardless of sex were divided into four groups according to initial weight and the average weight curves plotted with the results shown in Fig. 4.

Hence it seems to be established for rats of the same age, as previously shown by Osborne and Mendel for rats of different

ages, that the larger individuals have higher vitamin B requirement and so make smaller gains in weight if supplied with only the same limited amount of the vitamin. Upon complete deprivation of vitamin B, however, the larger animals survive, on the average, fully as long as the smaller ones of the same age.

When the records of all comparable cases were divided according to sex, it appeared that the average gain for the 8 weeks experimental period was: for forty-six males, 11.32 gm.; for thirty-eight females, 8.47 gm. From this it would appear that the

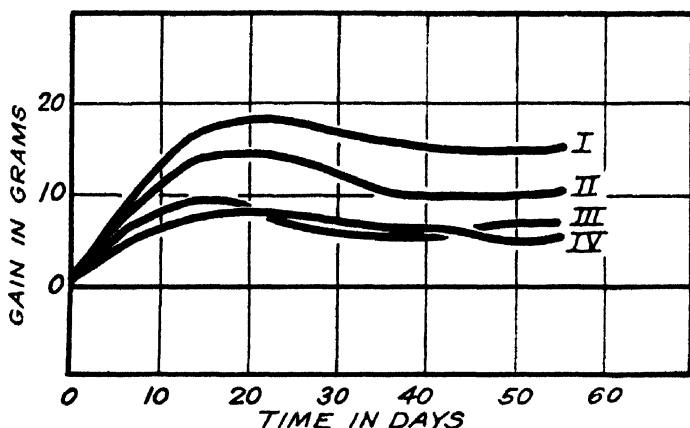


FIG. 4. Influence of initial size upon response to fixed allowance of vitamin B.

Curve	I.	Average gain curve of 19 rats, average initial wt. 34.63 gm.
"	II.	" " " " 22 " " " 45.77 "
"	III.	" " " " 21 " " " 54.29 "
"	IV.	" " " " 23 " " " 66.74 "

females, although weighing slightly less, have a slightly higher vitamin B requirement and therefore make slightly less gain upon the same limited intake of the vitamin. The difference in this case, however, is less than twice its probable error and may perhaps be accidental.

#### SUMMARY.

The experiments described deal with several of the factors involved in attempts to measure relative amounts of vitamin B by the rat growth method with as high a degree of quantitative accuracy as is possible.

The levels of feeding of vitamin B most conducive to accurate quantitative interpretation of results, and the form of the weight curve from week to week, are critically considered.

A preliminary "depletion period," so important in the case of vitamin A, did not increase the accuracy of the work with vitamin B.

Animals subjected to experiment at the uniform age of 4 weeks and receiving the same limited allowance of vitamin B, respond somewhat differently according to their size at the beginning of the experimental period, the larger animals making the smaller gains.

Under like conditions, males seem to make slightly larger gains than females, indicating that the latter have a slightly higher vitamin B requirement, at least when of equal size; but the difference between the averages for males and females was less than twice its probable error and may possibly have been accidental.



## VITAMIN B DETERMINATION AND REQUIREMENT WITH SPECIAL REFERENCE TO PROTEIN INTAKE.\*

BY H. C. SHERMAN AND O. H. M. GLOY.

(From the Department of Chemistry, Columbia University, New York.)

(Received for publication, May 7, 1927.)

Braddon and Cooper,<sup>1</sup> reporting in 1914 the results of their experiments with pigeons, emphasized the view that the amount of antineuritic substance required would be found to depend upon the amounts of the familiar organic foodstuffs ingested. Funk,<sup>2</sup> also as the result of work with pigeons, stated that as the proportion of protein in the diet increases the vitamin B requirement decreases; while, on the other hand, working with rats, Hartwell<sup>3</sup> and also Nelson<sup>4</sup> have held that a high protein diet increases the need for a liberal intake of vitamin B.

Both from the standpoint of nutritional requirements and of the determination of vitamin values of foods, it seemed important to settle the question whether a moderate change in the protein intake has any appreciable effect upon the vitamin B requirement. We have therefore experimented with rats in much the same manner that we should regularly proceed in the quantitative testing of foods for their vitamin B values; but have systematically varied the proportion of protein (casein) in the basal ration as well as the intake of vitamin B, furnished in these experiments in the form of orange juice.

\* Published as Contribution No. 546 from the Department of Chemistry, Columbia University.

<sup>1</sup> Braddon, W. L., and Cooper, E. A., *Brit. Med. J.*, 1914, i, 1348.

<sup>2</sup> Funk, C., Collazo, J. A., and Kaczmarek, J., *Compt. rend. Soc. biol.*, 1925, xcii, 997.

<sup>3</sup> Hartwell, G. A., *Biochem. J.*, 1921, xv, 140, 563; 1925, xix, 1074.

<sup>4</sup> Nelson, P. M., *J. Home Econom.*, 1926, xviii, 333.

## EXPERIMENTAL.

Healthy and normally growing young white rats were placed at 28 to 29 days of age in individual all metal cages with raised floors of wire screen to prevent access to excreta, and fed, with or without a fixed allowance of vitamin B in the form of orange juice, one of the following basal diets *ad libitum*.

Diet No.....	107A	107	107B	107C	107D
Purified casein.....	12	18	24	36	54
Butter fat.....	8	8	8	8	8
Cod liver oil.....	2	2	2	2	2
Osborne and Mendel salt mixture.....	4	4	4	4	4
Starch.....	74	68	62	50	32

All of the experimental animals were from families of the same strain which had been kept upon the same stock diet. Litters of six were ordinarily used and distributed evenly over each of three of the basal diets which were fed parallel, and with or without orange juice. Both males and females were used, but in the frequent repetitions of the experiments care was taken to secure an even distribution of the experimental animals with reference to sex as well as to initial size. The experimental periods were of 8 weeks duration. So far as its bearing upon the vitamin requirement of the animal is concerned, it should be emphasized that these experiments cover only a part of the period of rapid growth and do not touch upon the nutritional needs of reproduction and lactation. They are primarily concerned with the validity of our methods of testing vitamin values of foods.

*Series I.*—In this series basal diets of 12, 18, and 24 per cent protein were fed in strict parallel with each other and without vitamin B. The average survival period was 32.6 days with 12 per cent protein, 31.1 days with 18 per cent, and 32.1 days with 24 per cent casein, thus revealing no effect of the level of protein intake upon the ability to endure complete deprivation of vitamin B.

*Series II.*—Orange juice as sole source of vitamin B was fed in 1, 2, and 4 cc. portions (six times per week) to animals receiving basal diets containing 12, 18, and 24 per cent casein respectively,

the diet with 18 per cent casein being the one ordinarily fed in this laboratory in the testing of foods for vitamin B values. At

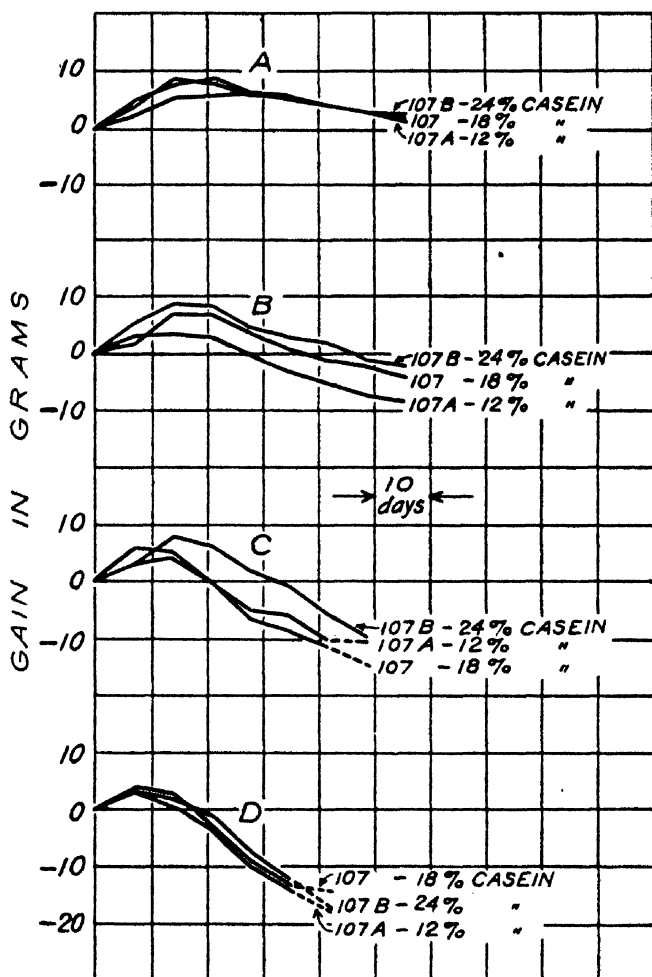


FIG. 1. The average gain curves of animals fed on basal diets varying in protein content from 12 to 24 per cent and the various portions of orange juice. A gives the results for Diets 107B (24 per cent casein), 107 (18 per cent casein), 107A (12 per cent casein), and 4 cc. of orange juice; B for the same diets and 2 cc. of orange juice; C for the same diets and 1 cc. of orange juice; D for the basal diets only. The dashed portion of the curves indicates that less than half the animals were surviving.

each level of vitamin intake, the three basal diets containing respectively 12, 18, and 24 per cent casein gave essentially the same results.



Fig. 1 summarizes graphically the average result for each level of vitamin feeding and of protein intake in the first and second series of experiments.

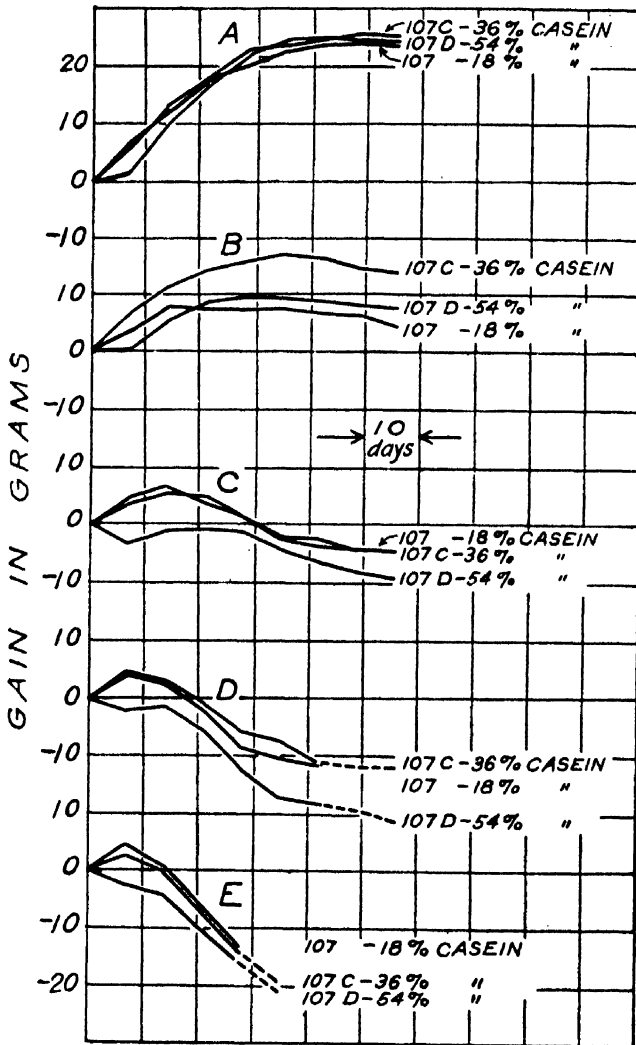


FIG. 2. The average gain curves of animals fed on basal diets varying in protein content from 18 to 54 per cent and the various portions of orange juice. A gives the results for Diets 107 (18 per cent casein), 107C (36 per cent casein), 107D (54 per cent casein), and 6 cc. of orange juice; B for the same diets and 4 cc. of orange juice; C for the same diets and 2 cc. of orange juice; D for the same diets and 1 cc. of orange juice; E for the basal diets only. The dashed portion of the curves indicates that less than half the animals were surviving.

*Series III.*—Here animals receiving no vitamin B were compared as to their survival periods on basal diets of 18, 36, and 54 per cent casein (Diets 107, 107C, 107D). The average results were 26.0, 26.7, and 26.3 days respectively, showing no influence from even the high levels of protein here used. (That the survival period on the standard level of 18 per cent casein was somewhat shorter in Series III than in Series I is evidently due to change of season or some other cause affecting all the animals of Series III alike.)

*Series IV.*—Here parallel groups of animals on basal diets containing 18, 36, and 54 per cent casein respectively, were fed at four different levels of vitamin B intake, namely with 1, 2, 4, and 6 cc. of orange juice daily except Sundays. Again at each level of vitamin intake the results were essentially the same for the three different levels of protein intake showing that even the very high protein diets did not appreciably influence the vitamin B requirement as reflected in the response of the weight curve to a given restricted allowance of vitamin B. (It was observed incidentally that the general condition of the animals on the very high protein diets was not so good as that of those receiving the usual proportion of 18 per cent.)

\* The average results for each combination of protein and vitamin feeding in Series III and IV are summarized graphically in Fig. 2.

In order to economize space all tabulations of numerical data are here omitted.

#### SUMMARY AND CONCLUSIONS.

In a diet devoid of vitamin B but adequate in all other respects, the proportion of protein (casein) was varied from 12 to 54 per cent without appreciably influencing the ability of the experimental animals to survive the deprivation of the vitamin.

The same variations of protein intake also failed to show any difference in the response of the weight curves of experimental animals receiving uniform restricted allowances of vitamin B in the form of orange juice. This was found both in the case of maintenance and of submaintenance allowances of the vitamin.

No basis is found for any belief that the vitamin B requirement

is influenced by the protein intake in the age period covered by these experiments.

The results amply justify the conclusion that basal diets may vary widely in protein content and still yield interchangeable results in the testing of foods for vitamin B; and also that with a basal diet containing 18 to 20 per cent of protein, the protein intake may be much changed by the feeding of either a high protein or a low protein food as a source of vitamin B without affecting the validity and quantitative value of the test as an indication of the relative vitamin B content of the food tested.

## FORMATION OF LACTIC ACID IN THE BODY AFTER SEVERE HEMORRHAGE.

By CECILIA RIEGEL.\*

(From the Department of Physiological Chemistry, School of Medicine,  
University of Pennsylvania, Philadelphia.)

(Received for publication, May 3, 1927.)

It has been demonstrated that there may occur after severe hemorrhage a decrease in pH and in alkaline reserve of the blood (Bennett, 1926; Evans, 1921; Milroy, 1917). Such changes may be brought about either by a diffusion of base from blood to tissues, or by increased production of fixed acid. According to Haggard and Henderson (1922) diffusion of base from blood to tissues occurs as a compensatory reaction for increased alkalinity produced by hyperventilation. However, while we might picture enough base passing to the tissues to maintain the pH of the blood at a normal level, it does not seem probable that there should be a transfer of base sufficiently great to cause a diminished pH. On the other hand, increased production of fixed acid, such as lactic acid, in the tissues, and its diffusion into the blood, might readily account for increased acidity and decreased alkaline reserve in the blood. To determine, therefore, whether an increase in concentration of lactic acid in the blood occurs under these conditions, dogs were subjected to severe hemorrhages, and blood analyzed at various intervals thereafter.

### *Methods.*

Normal dogs were used. Blood, with one or two exceptions, was taken from the heart by means of lumbar puncture needles, 16 to 17 gauge. The dogs were not anesthetized and usually remained quiet. For the hemorrhage 30 to 47 per cent of the total blood volume was removed, calculating total blood volume

\* Denison Medical Foundation Fellow, 1925-26. Alpha Xi Delta Fellow, American Association of University Women, 1926-27.

as 8 per cent of the body weight. The hemorrhage lasted for approximately 15 to 45 minutes. Samples for analysis—about 20 cc.—were taken in a centrifuge tube under oil. The tube contained sodium oxalate and sodium fluoride in amounts sufficient to make respectively a 0.4 and a 0.1 per cent solution in the blood (Evans, 1922).

Hematocrit determinations were made on oxalated blood, using Daland hematocrit tubes. Total carbon dioxide was determined by the method of Van Slyke and Stadie (1921), using the Van Slyke volumetric apparatus.

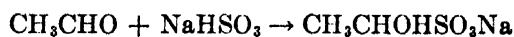
### *Lactic Acid.*

The method used was essentially Clausen's sulfuric acid method (1922). A careful study of the method was made and certain changes in the details of the method as outlined by Clausen were found helpful in shortening the time necessary for the procedure and in obtaining consistent results. As the descriptions published by those who have used the method are very meager in details, making it more or less difficult to set up the apparatus and carry out the determination, a complete description of the procedure followed may not be out of place.

The solution containing the lactic acid is heated with sulfuric acid, which converts the lactic acid to acetaldehyde.



The aldehyde so formed is distilled by means of a current of steam and air into sodium bisulfite solution. The aldehyde combines with the sodium bisulfite.



The excess of bisulfite is then titrated with iodine. Subsequently the bisulfite-aldehyde compound is decomposed by adding sodium bicarbonate, and the bisulfite so liberated titrated with iodine. From this second titration can be calculated the amount of acetaldehyde, and therefore the amount of lactic acid originally present.

### *Apparatus.*

The oxidation of lactic acid was carried out in an ordinary Pyrex tube, 25 × 250 mm., fitted with a three-hole rubber stopper.

In the stopper were a thermometer and a glass inlet tube, both of which dipped under the liquid, and also an outlet tube which was connected with an air condenser, about 16 inches in length. The tube was immersed to a depth of about 2 inches in a bath of Wood's metal (melting point below  $100^{\circ}$ ), contained in an agate-ware cup. Four tubes could be accommodated in this vessel, thus allowing four determinations to be made at one time. Two receiving tubes, also  $25 \times 250$  mm., were used for each determination, each containing 20 cc. of approximately 0.1 N sodium bisulfite. The inlet tubes in the receiving tubes were of the type recommended for blood urea and blood ammonia determinations, having at the end a bulb with four to five small holes. The receiving tubes were immersed in a constant level water bath, through which cold water circulated. From a suction pump a current of air of approximately 2000 cc. per minute passed through each set of tubes.

#### *Procedure.*

A Folin-Wu tungstic acid filtrate was made and sugar removed from this filtrate by the method of Van Slyke (1917). 5 cc. of this filtrate were pipetted into the reaction tube, the air current turned on, 10 cc. of 50 per cent sulfuric acid added, and the tubes immersed in the bath. The distillation was continued for 15 minutes after the temperature of the reaction mixture (rather than that of the metal bath, as suggested by Clausen) reached  $140^{\circ}\text{C}$ . The temperature was maintained between  $140$ – $145^{\circ}\text{C}$ . Charring may occur if the temperature rises above  $150^{\circ}$ . From time to time water from a wash bottle was spurted through the inlet tube into the reaction mixture to prevent the acid becoming too concentrated and to keep the temperature from rising. When the distillation was complete, the air current was discontinued and the contents of the receiving tubes washed into an Erlenmeyer flask. By cooling the receiving tubes in cold water practically all the aldehyde was retained in the first receiving tube.

In the titration the uncombined sodium bisulfite was titrated with approximately 0.1 N iodine to near the end-point, and the actual end-point was reached by the use of 0.001 N iodine. Soluble starch was used as an indicator. It was prepared by putting 5 gm. of potato starch in 1 liter of boiling water. After standing 18

# 126      Blood Lactic Acid after Hemorrhage

hours, this was filtered, and about 2 cc. of filtrate were used for each determination. After the first end-point was reached, 3.5

TABLE I.

Concentration of Zn lactate solution.	Amount taken.	Lactic acid taken.	Time of heating.	Temperature.	0.001 N iodine corrected for blank, 0.37 cc.	Lactic acid found.	Yield.	Average yield.
<i>N</i>	cc.	mg.	min.	°C.	cc.	mg.	per cent	per cent
0.0025	1	0.225	15	140-145	4.90	0.220	98.0	97.7
					4.85	0.218	96.9	
					4.85	0.218	96.9	
					4.87	0.219	97.6	
					4.94	0.222	98.8	
					4.92	0.221	98.4	
0.0025	1	0.225	25	140-145	4.85	0.218	96.9	97.7
					4.88	0.219	87.6	
					4.89	0.220	97.7	
					4.93	0.221	98.9	
					4.90	0.220	98.0	
0.0025	1	0.225	35	140-145	4.90	0.220	98.0	97.9
					4.84	0.217	96.8	
					4.95	0.222	98.9	
0.001	5	0.450	15	135-140	4.20	0.378	42.0	43.0
					4.00	0.360	40.0	
					3.80	0.342	38.0	
					5.20	0.468	52.0	
0.001	5	0.450	30	135-140	9.72	0.437	97.0	97.2
					9.77	0.439	97.5	
0.001	5	0.450	15	140-145	9.67	0.435	96.7	97.1
					9.77	0.439	97.6	
0.05	1	4.500	15	140-145	9.38	4.220	93.8	93.0
					9.27	4.170	92.6	
					9.30	4.180	92.8	
0.05	1	4.500	30	135-140	8.61	3.870	86.0	86.8
					8.59	3.860	85.7	
					8.95	4.000	88.8	

to 4.0 cc. of a saturated solution of sodium bicarbonate were added, and the bisulfite thus liberated from the aldehyde-bisulfite com-

pound was titrated with 0.001 N iodine (in the case of blood analysis). The end-point was taken as the point where addition of another 0.5 cc. of sodium bicarbonate did not cause immediate decolorization. When the end-point is reached the blue color persists for at least 30 seconds. The blank with 0.001 N iodine was 0.37 cc.

It was found that by heating for 15 minutes at 145° and using a vigorous air current, a 97 to 98 per cent yield of lactic acid was obtained with a standard zinc lactate solution (Table I). Longer distillation did not increase the yield (Table I). This is a decided shortening of the time specified by Clausen, who considered it necessary to distil for 1 hour, and by McGinty and Gesell (1925), who distilled for 2 hours at 150°C. No charring resulted from heating the reaction mixture to 145°C. With lower temperature the yield was considerably lower in 15 minutes (Table I). Distilling for 30 minutes at 135–140° was hardly sufficient to give good results. When extremely large amounts of lactic acid are to be determined the time of distillation must be increased. Such large amounts of lactic acid as were determined here (see Table I) are not met with in blood, and the necessity of determining too large amounts may be avoided altogether by taking smaller aliquot portions of the blood filtrate for analysis. If, on the other hand, very small amounts of lactic acid are to be determined, a larger portion of the filtrate may be used, and a more concentrated sulfuric acid added.

Care should be taken in weighing the zinc lactate as it is slightly hygroscopic and a considerable error may be introduced here.

Commercial preparations of sodium bisulfite were unsatisfactory and gave poor recovery of acetaldehyde. The sodium bisulfite was always prepared by conducting sulfur dioxide into a saturated sodium bicarbonate solution until carbon dioxide was no longer given off. Bisulfite so prepared gave the reactions to indicators (alkaline to methyl orange, acid to methyl red) suggested by Cajori, Crouter, and Pemberton (1924). This concentrated solution, which did not deteriorate over a period of several months, was diluted as needed.

To decompose the aldehyde-bisulfite compound, it was necessary to use an excess of sodium bicarbonate solution rather than just sufficient to decolorize the solution; otherwise too low results



were obtained, due to the fact that the blue color persisted in the solution and faded only slowly, long before the true end-point of the reaction was reached. Usually 1 to 2 cc. of sodium bicarbonate (saturated solution) were sufficient for decolorization. 1 to 2.5 cc. were added in excess of this amount, making a total of 3.5 cc. Traces of sodium carbonate in the sodium bicarbonate were not harmful, although larger amounts (0.1 per cent) were found to cause fairly rapid fading of the blue color so that too high titration values were obtained.

#### DISCUSSION.

Lactic acid is produced in tissues in excessive amounts only in the absence of an adequate oxygen supply (Fletcher and Hopkins, 1907). After hemorrhage there necessarily is a diminished oxygen supply, due to reduction in the amount of hemoglobin, and to decreased volume flow of blood (Gesell and Moyle, 1922). This will cause excessive formation of lactic acid in the tissues. It has been shown by the experiments of Hill, Long, and Lupton (1924) and Barr, Himwich, and Green (1923) on exercise, that lactic acid readily passes from tissues to blood. After hemorrhage, therefore, the excess lactic acid formed in the tissues will presumably diffuse into the blood, the amount in the blood depending upon the amount formed in the tissues.

Results of the experiments are given in Tables II to V. The data on production of lactic acid after hemorrhage show that there is a comparatively quick rise in concentration of lactic acid in the blood, followed by a gradual return to normal. Extent of hemorrhage is apparently the factor most concerned in determining the amount of increase in lactic acid and the time during which this increase shall continue. We may say that the larger the hemorrhage, the greater the increase in lactic acid in the blood will be. Also, with the same amount of bleeding the lactic acid in the blood increases to approximately the same level. For example, Hemorrhage 1 on Dog 1 and Hemorrhage 2 on Dog 4 were of about the same magnitude, and were not extreme bleedings. Lactic acid in the blood rose to about the same level after bleeding and returned to normal in the same time. In Hemorrhage 1, Dog 1, 31 per cent of the calculated blood volume was removed, and lactic acid increased to 35 mg. per 100 cc. at the

TABLE II.

Dog 1. Brown and white female. Weight 10 kilos.

Hemorrhage 1.		Hemorrhage 2.		Hemorrhage 3.		Hemorrhage 4.		Remarks.
Time.	Lactic acid. mg. per cent	Time.	Lactic acid. mg. per cent	Time.	Lactic acid. mg. per cent	Time.	Lactic acid. mg. per cent	
May 10 10.00 a.m.	18.0	May 13 9.00 a.m.	19.8	May 18 9.00 a.m.	19.5	June 3 9.30 a.m.	21.9	In Hemorrhage 1, 250 cc. or 31 per cent blood volume removed between 9.40-9.55 a.m. In Hemorrhage 2, 350 cc. or 43 per cent blood volume removed between 9.05-9.30 a.m. In Hemorrhage 3, 300 cc. or 37 per cent blood volume removed between 9.15-9.45 a.m. In Hemorrhage 4, 325 cc. or 40 per cent blood volume removed between 9.30-9.50 a.m.
May 11 9.55 a.m.	35.4	9.30 "	35.1	9.45 "	36.6	9.50 "	39.0	
10.09 "	30.3	9.50 "	44.1	11.00 "	36.6	10.15 "	57.1	
10.25 "	21.6	10.30 "	47.4	12.00 m.	33.3	10.45 "	39.9	
11.00 "	19.5	11.05 "	55.0	May 19 12.00 m.		12.00 m.	33.0	
		May 14 3.00 p.m.	30.6	9.00 a.m.	20.1	2.00 p.m.	30.6	
						June 4 10.45 a.m.	24.6	
						2.00 p.m.	25.5	

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TABLE III.

Dog. 2. Brown female collie. Weight 10.8 kilos.

Time.	Lactic acid.	Carbon dioxide.	Hemato-crit.	Remarks.
	<i>mg. per cent</i>	<i>vol. per cent</i>	<i>per cent</i>	
June 9				Hemorrhage 1.
9.05 a.m.	39.6	51	59	Between 9.05-10.45 a.m. 400
10.45 "	66.0			cc. or 47 per cent blood
11.05 "	69.9			volume removed.
11.35 "	77.7			
1.00 p.m.	52.2	45	50	
June 10				
9.10 a.m.	27.3	63	40	
June 11				Hemorrhage 2.
9.05 a.m.	80.7	64	38	Between 9.30-10.15 a.m. 315
10.30 "	168.9	49	35	cc. or 36 per cent blood
11.00 "	162.0	40	28	volume removed.
12.15 p.m.	162.0			Shortly before 3.00 p.m. dog
3.00 "	215.0			showed signs of considerable
3.05 "	219.0	23	28	distress and weakness. Died
				while blood was being taken
				at 3 p.m.

TABLE IV.

Dog 3. White female. Weight 12.2 kilos.

Time.	Lactic acid.	Carbon dioxide.	Hemato-crit.	Remarks.
	<i>mg. per cent</i>	<i>vol. per cent</i>	<i>per cent</i>	
June 22				Hemorrhage 1.
9.30 a.m.	24.9	53	48	Between 10.10-10.40 a.m. 475
10.40 "	34.8	49	48	cc. or 46 per cent blood
11.00 "	41.4		47	volume removed.
11.30 "	39.0	52	47	
12.30 p.m.	32.7		40	
2.30 "	27.8	56	41	
June 23				
11.00 a.m.	21.6	67	30	
2.30 p.m.	33.3*	59	27	

\* No duplicate.

end of bleeding, with a return to normal in 30 minutes. In Hemorrhage 2, Dog 4, 33 per cent of the calculated blood volume

was removed, and lactic acid increased to 38 mg. per 100 cc. after bleeding and was again normal in 40 minutes. In both cases lactic acid in the blood reached its highest value just at the end of bleeding and began to decrease again as soon as bleeding was stopped.

TABLE V.

Dog 4. Brown male. Weight 13.2 kilos.

Time.	Lactic acid.	Carbon dioxide.	Hemato-crit.	Remarks.
	<i>mg. per cent</i>	<i>vol. per cent</i>	<i>per cent</i>	
Nov. 2				Hemorrhage 1.
10.40 a.m.	33.6	53	45	Between 10.40-11.00 a.m. 275
10.45 "	36.0	51	45	cc. or 26 per cent blood
10.55 "	39.0	51	45	volume removed.
11.40 "	40.8	48	43	
1.20 p.m.	34.5	50	43	From 1.20-1.25 p.m. 100 cc.
1.25 "	38.7	51	43	removed.
Nov. 20				Hemorrhage 2.
10.55 a.m.	25.8	49		Between 10.55-11.15 a.m. 350
11.15 "	38.2			cc. or 33 per cent blood
11.35 "	31.9	44		volume removed.
12.00 m.	26.7	38		
2.00 p.m.	22.8	45		
Dec. 1				
1.15 p.m.	16.8	52		
Dec. 14				Hemorrhage 3.
10.15 a.m.	35.7	49		Between 10.15-10.50 a.m. 400
10.50 "	44.7	48		cc. or 37 per cent blood
11.05 "	49.2	44		volume removed.
11.45 "	54.3	39		
12.45 p.m.	39.0	44		
2.00 "	32.4	48		
Dec. 15				
2.00 p.m.	31.8			

With somewhat more severe bleeding lactic acid concentration in the blood reached a higher level, and also continued to increase for some time after bleeding stopped. In Hemorrhage 2 on Dog 1, 43 per cent of the calculated blood volume was removed. The lactic acid in the blood at the end of bleeding was 38 mg. per 100 cc., and continued to increase for 1 hour and 15 minutes, reaching

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55 mg. per 100 cc. This experiment has a parallel in Hemorrhage 3 on Dog 4, where 39 per cent of the calculated blood volume was removed. Lactic acid rose to 44 mg. per 100 cc. at the end of bleeding and continued to increase for 1 hour after hemorrhage stopped, reaching 54 mg. per 100 cc.

Dog 2 was subjected to a very severe first bleeding (47 per cent of the blood volume removed), and lactic acid in the blood increased from 39.6 to 66 mg. per 100 cc. at the end of bleeding. Lactic acid continued to increase in the blood for 90 minutes after bleeding was stopped, reaching a concentration of 77 mg. per 100 cc. This experiment was the most severe of those reported, and the lactic acid concentration in the blood reached a higher level, and the total increase was greater, than in any other experiment. Although the hemorrhage was a severe one the animal apparently recovered and lactic acid on the following day was 27 mg. per 100 cc. This recovery, however, was only temporary, because 2 days after Hemorrhage 1 the lactic acid was extremely high—80 mg. per 100 cc. As the animal did not struggle when blood was taken, this high lactic acid is not due to excessive muscular activity. When the animal was again deprived of a considerable portion of its blood volume the hemorrhage was fatal (Hemorrhage 2, Dog 2). Although in a normal animal removal of 36 per cent of the blood volume would not be extreme, this animal, being in a weakened condition, was not able to survive. Lactic acid in the blood rose at the end of the second bleeding from 80 to 168 mg. per 100 cc. It remained at this level for about 2 hours. Later, however, the concentration of lactic acid again increased, and just before death was 215 mg. per 100 cc.

Return of lactic acid in the blood to normal after hemorrhage is much more prolonged than the increase. Rapid production and delayed removal of lactic acid are also found in severe exercise, as shown by Hill, Long, and Lupton (1924). These workers have shown that after a few minutes of severe exercise, when lactic acid in the blood is 100 to 200 mg. per 100 cc., 80 to 120 minutes are required for complete recovery. The similarity of the results of the hemorrhage experiments to those on exercise suggests that there also lactic acid is removed by the same process as the lactic acid produced in exercise; namely, synthesis to glycogen. The return of lactic acid to normal after hemorrhage will be a slower

process than after exercise, due to the fact that lactic acid may continue to be produced in abnormal amounts for some time after hemorrhage is stopped, whereas in exercise lactic acid production ceases when the muscle stops contracting. It is interesting to note that in every case (except where hemorrhage terminated in death) on the day following the hemorrhage the concentration of lactic acid in the blood is again normal, indicating that for the animal at rest the oxygen supply is adequate, although the amount of hemoglobin is diminished.

Total carbon dioxide also is decreased following hemorrhage. As Bennett (1926) found the carbon dioxide tension following hemorrhage was normal or above normal, a study of total carbon dioxide probably gives an approximate indication of the alkaline reserve. We may say, therefore, that the alkaline reserve is decreased at the same time that the concentration of lactic acid is increased above normal.

The experiments reported above give evidence against the theory of Haggard and Henderson that in the anoxemia following hemorrhage the decrease in alkaline reserve is due to diffusion of base from the blood to the tissues, and not to production of lactic acid. However, Haggard and Henderson have many times emphasized the fact that there is anoxemia following hemorrhage. The production of lactic acid in excess amounts after hemorrhage, as demonstrated in the experiments reported above, is additional evidence in support of the view that anoxemia is a result of heavy hemorrhage. On the day following hemorrhage, however, although hemoglobin is still below normal, apparently the oxygen supply to the tissues is adequate for an animal at rest, as indicated by the fact that the concentration of lactic acid in the blood is always normal.

#### SUMMARY.

After severe hemorrhages of 30 to 46 per cent of the total blood volume (calculated as 8 per cent of the body weight) the concentration of lactic acid in the blood is increased, the total increase and duration of the increase depending upon the extent of the hemorrhage.

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The writer wishes to express her sincere appreciation to Dr. D. Wright Wilson for many suggestions and criticisms during the course of the work reported in both this and the following paper.

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# THE RATE OF DISAPPEARANCE OF SODIUM LACTATE INJECTED INTRAVENOUSLY AND ITS EFFECT UPON SUGAR AND INORGANIC PHOS- PHATE OF THE BLOOD.

BY CECILIA RIEGEL.\*

(From the Department of Physiological Chemistry, School of Medicine,  
University of Pennsylvania, Philadelphia.)

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Since the fundamental work of Fletcher and Hopkins on the formation of lactic acid in muscle, considerable knowledge has been gained concerning the chemical changes taking place in contraction and in recovery of muscle. Embden and coworkers (1914, 1917, 1921) and Meyerhof (1920) have shown the nature of the precursors of lactic acid and the reactions which during contraction lead to the production of lactic acid.

During muscle contraction inorganic phosphates and lactic acid are formed in equimolecular amounts. At the same time the glycogen of muscle is diminished (Meyerhof, 1920). Embden showed that the hexose diphosphate obtained from yeast fermentation can also be converted to lactic acid and phosphate by muscle extract, and the same substance added to a contracting muscle causes increased lactic acid formation. By adding phosphates to muscle under anaerobic conditions all the glycogen can be converted to lactic acid. Embden therefore concluded that there is in muscle a compound similar to the hexose diphosphate formed in yeast fermentation. This substance is the precursor of lactic acid and phosphate, and may itself be formed from glycogen, with utilization of phosphate. He has isolated such a substance from muscle, and showed that the compound, which he calls lactacidogen, is probably identical with the hexose diphosphate of yeast fermentation. Meyerhof

\* Denison Medical Foundation Fellow, 1925-26. Alpha Xi Delta Fellow, American Association of University Women, 1926-27.



(1920) has further extended the work and shown that, in recovery from contraction, four-fifths or perhaps all of the lactic acid formed is reconverted to glycogen. The energy for this reversion is derived from the burning of one-fifth of the lactic acid, or its equivalent of glucose produced from glycogen.

We also have evidence, in the work of Hill, Long, and Lupton (1924) on exercise, that lactic acid may be produced as the result of contraction of muscle in the body itself, and that removal of the lactic acid so produced is brought about by reversion to lactacidogen. This same process probably occurs in the removal of lactic acid produced in other abnormal phases of metabolism. After severe hemorrhage lactic acid in the blood is increased, probably as the result of a deficient oxygen supply to the tissues, and, with recovery, disappears at a rate similar to that observed by Hill, Long, and Lupton in their exercise experiments.

It has not been established whether this same mechanism would operate to remove lactic acid introduced into the body from an outside source. It was therefore with the idea of obtaining some information concerning the process of removal that experiments on the injection of sodium lactate were undertaken.

#### EXPERIMENTAL.

The data from eight experiments on dogs are reported below. Five experiments were made on normal dogs, three of which fasted for 7 days before the experiments, and two of which were fed regularly until the experimental day, during which no food was given until after collection of blood samples had been completed. One experiment was on an anemic dog, which also was fed regularly, and two experiments on a depancreatized animal. The diet in all cases was a mixed one. The fasting animals received water by stomach tube daily.

A hypertonic solution of sodium lactate was injected. The solution was prepared by neutralizing c.p. lactic acid (approximately 85 per cent) with 50 per cent sodium hydroxide. The solution contained the equivalent of 303 mg. of lactic acid per cc. in both experiments on Dog 1, and of 425 mg. of lactic acid per cc. in all other experiments. The different amounts injected were 606, 850, 1725, and 2125 mg. While the injection of 2.1 gm. of lactic acid may seem to be excessive, actually it does not nearly

approach the amounts of lactic acid which are dealt with by the body after exercise. According to Hill, Long, and Lupton (1924) in severe exercise 2 to 3 gm. per kilo per minute may be produced.

Injections were made into the jugular vein, and blood samples removed either from the vein or from the heart. Blood was collected under oil in 50 cc. centrifuge tubes containing sodium fluoride and potassium oxalate, as described by Evans (1922). Not more than 20 cc. of blood was removed at each bleeding. No anesthetic was given, and animals usually remained quiet while blood was taken and the sodium lactate injected.

Blood sugar was determined by the method of Folin and Wu (1919) a tungstic acid filtrate being used. Lactic acid was determined by Clausen's sulfuric acid method (1922) with the slight modifications discussed in the previous paper. Inorganic phosphates were determined in a trichloroacetic acid filtrate by the method of Fiske and Subbarow (1925). Carbon dioxide was determined by the method of Van Slyke and Stadie (1921).

Urine was collected for the 24 hours preceding the day of experiment, and also on the experimental day. In the early experiments the animals were catheterized in order to have complete separation of the urine for each day, and in some cases the animals were catheterized several times on the experimental day in order to obtain urine at different periods after the injection of sodium lactate. Later, catheterization was not performed, the urine which was naturally voided being collected. Lactic acid in urine was determined by Clausen's method.

The values for sugar, inorganic phosphates, and lactic acid in blood taken before injections are within the normal range of values reported in the literature.

The data show that in both the normal and diabetic animals two-thirds of the lactic acid had disappeared from the blood in 5 to 10 minutes after injection. Approximately 30 minutes after the injection the rate of removal of lactic acid from the blood became much slower. Normal values were not obtained for 1 to 2 hours. Inorganic phosphates in normal dogs showed an immediate decrease, the extent of the decrease depending upon the amounts of sodium lactate injected. In two of the experiments on normal dogs blood sugar showed an insignificant rise.

In three experiments, where the larger doses of sodium lactate were used, the blood sugar was definitely increased. This rise in sugar is a delayed one, occurring only when the lactic acid is approaching normal. With Dog 5, which struggled during the injection, blood sugar showed an immediate and marked increase, probably the result of excitement. In the same experiment, although a large amount of lactic acid was given, inorganic phosphates showed only a slight fall. However, this is probably significant in view of the fact that struggling should cause an increase in blood phosphates.

TABLE I.

Dog 1. Weight 5 kilos. Depancreatized. Given insulin until 3 days before experimental day.

Time.	Lactic acid.	Inorganic P.	Remarks.
	<i>mg. per cent</i>	<i>mg. per cent</i>	
May 27			
9.30 a.m.	24.9	6.4	At 9.51 a.m. 2 cc. sodium lactate equivalent to 606 mg. lactic acid injected into jugular vein.
9.56 "	66.0	0.0	
10.25 "	39.6	5.9	
11.10 "	36.3	0.0	
12.00 m.	24.4	5.7	
June 7			
9.30 a.m.	50.7		At 10.10 a.m. 2 cc. sodium lactate solution equivalent to 606 mg. lactic acid injected into jugular vein.
10.15 "	130.0		
10.45 "	81.3		
11.20 "	69.6		
12.00 m.	66.0		

## DISCUSSION.

The data reported in Tables I to V on lactic acid in the blood indicate that there is a rapid disappearance of the lactic acid from the blood after the injection of sodium lactate. Within 5 minutes after the injection of an amount of sodium lactate equivalent to 850 mg. of lactic acid, the blood shows a concentration of approximately 60 mg. of lactic acid per 100 cc., or 40 mg. above the average normal concentration of 20 mg. per 100 cc. Assuming an average weight of 10 kilos, and calculating the blood volume as 8 per cent of the body weight, we should expect to find, if the 850 mg. were evenly distributed throughout the blood, 126 mg. of lactic acid

per 100 cc. of blood. In 5 minutes, therefore, two-thirds of the lactic acid injected has disappeared.

A possible explanation for this rapid decrease is that the in-

TABLE II.

Dog 2. Weight 10 kilos. Previously used for experiments on hemorrhage. Anemic but otherwise normal. Received food up to the day of experiment.

Time.	Lactic acid.	Glucose.	Inorganic P.	Remarks.
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	
June 15				
9.15 a.m.	31.5	86	4.8	At 10.15 a.m. 606 mg. lactic acid as sodium lactate injected into jugular vein. Subsequent samples taken from jugular vein.
10.20 "	59.0	86	4.3	
11.50 "	45.3	86	4.2	
1.00 p.m.	32.4	84	4.2	
2.00 "	34.8	86	4.1	

TABLE III.

Dog 3. Weight 12 kilos.

Time.	Lactic acid.	Glucose.	Inorganic P.	Remarks.
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	
Nov. 16				
10.30 a.m.	22.2	65	4.2	Dog received no food Nov. 9-15. At 11.05 a.m., Nov. 16, 2 cc. sodium lactate solution equivalent to 850 mg. lactic acid injected into jugular vein. Subsequent samples from jugular vein.
11.10 "	68.6	66	4.0	
11.30 "	39.2	66	3.8	
11.45 "	37.8	67	3.6	
12.15 p.m.	22.2	68	3.7	
Jan. 18				
10.15 a.m.	27.9	82	5.3	Between 10.25-10.35 a.m., 3 cc. sodium lactate solution equivalent to 1275 mg. lactic acid injected into jugular vein. Subsequent samples from heart.
10.45 "	58.8	82	4.3	
11.05 "	46.2	82	4.0	
11.30 "	35.1	85	4.0	
12.30 p.m.	29.1	85	3.8	
2.00 "	21.3	90	4.7	

jected lactic acid is distributed throughout the total water of the body. A consideration of the data shows that about 25 minutes after the injection of sodium lactate a change occurs in the rate of removal of the lactic acid from the blood, and it

TABLE IV.

Dog 4. Weight 10.4 kilos.

Time.	Lactic acid.	Glucose.	Inorganic P.	Remarks.
	mg. per cent	mg. per cent	mg. per cent	
Dec. 7				
10.45 a.m.	16.8	83	3.6	Dog received no food Nov. 30-Dec. 7.
11.00 "	51.8	81	3.5	From 10.48-10.50 a.m., Dec. 7, 2 cc.
11.15 "	42.0	84	3.0	sodium lactate solution equivalent to
12.00 m.	37.8	86	2.8	850 mg. lactic acid injected into jugular vein.
1.05 p.m.	21.6	102	2.4	Subsequent samples taken from heart.
Nov. 11				
10.15 a.m.	15.3	70	3.1	Dog received no food from Nov. 4-11.
10.45 "	60.4	74	1.2	5 cc. sodium lactate solution equivalent to 2125 mg. lactic acid were injected into jugular vein between 10.30
11.00 "	40.3	68	0.0	-10.35 a.m., Nov. 11. Subsequent
11.30 "	35.7	78	1.7	samples taken from heart.
12.30 p.m.	18.9	80	0.8	
1.35 "	12.0	79	0.7	
2.30 "	14.7	91	0.6	

TABLE V.

Dog 5. Brown and black female. Weight 8.56 kilos.

Time.	Lactic acid.	Glucose.	Inorganic P.	Remarks.
	mg. per cent	mg. per cent	mg. per cent	
Feb. 1				
10.45 a.m.	29.1	89	2.0	At 10.50 a.m. 1 cc. sodium lactate equivalent to 425 mg. lactic acid injected into jugular vein. Dog very excited and moved, so needle was pulled out of vein. Subsequently 4 cc. sodium lactate equivalent to 1680 mg. lactic acid injected into femoral artery, exposed under cocaine. Blood samples for analysis from heart.
11.35 "	88.2	136	1.7	
11.40 "	77.0	143	1.7	
11.55 "	51.8	166	1.7	
12.25 p.m.	36.4	143	1.6	
1.30 "	28.8	90	2.1	

was thought that this might represent the time when lactic acid had become uniformly distributed in the body fluids. If we assume 80 per cent of the body weight is water, we can calculate

what concentration of lactic acid would result from the injection of various amounts of sodium lactate into different animals. In Table VI are given the calculated values for blood lactic acid after injection of different amounts of sodium lactate, assuming all the lactic acid to be distributed uniformly throughout the body fluids. There are also given the observed values found at the time when the change in rate of removal occurred.

In four of the six experiments which could be compared in this way the observed and calculated values show close agreement. In a calculation of this sort only a roughly quantitative comparison

TABLE VI.

	Amount injected.	Time after injection.	Lactic acid.		Deviation of observed from calculated values.
			Calculated.*	Observed.	
	<i>mg.</i>	<i>min.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Dog 1.					
Experiment 1.	606	35	40.0	39.6	0.4
" 2.	606	35	65.8	81.3	16.5
Dog 3.					
Experiment 1.	850	25	31.0	39.2	8.2
" 2.	1275	30	41.1	46.2	5.1
Dog 4.					
Experiment 1.	850	25	27.0	42.0	15.0
" 2.	2125	25	40.8	39.2	1.6

\* Calculated values represent the initial lactic acid in blood as actually determined before the experiment, plus the amount injected per 100 cc. of body fluid.

can be made because of certain complicating factors. While diffusion from the blood is going on, there is also taking place removal of lactic acid by conversion to glycogen, as will be discussed later. This would tend to make the observed values lower than the calculated values, as the latter do not take into account the removal of some lactic acid by the second process. On the other hand, the rate of diffusion would decrease as the concentration of lactic acid in the blood and other fluids approached equality. This would tend to make the observed values higher than the calculated ones. These two factors are working in opposite directions and would therefore to some extent balance

each other. It seems permissible to conclude from the data that the first rapid disappearance is due for the most part to a diffusion of lactic acid from the blood to other fluids until equal concentration is approached. Subsequently the rate of diffusion of lactic acid from the blood to other body fluids will depend upon the rate of removal of lactic acid from these fluids.

Normally lactic acid is present in tissues only in very small amounts. At rest muscle is alkaline to litmus, becoming acid when working. The concentration of lactic acid in resting muscle is stated by Fletcher and Hopkins (1907) to be 15 mg. per 100 gm., but this amount, according to them, represents the "unavoidable minimum" due to irritation and stimulation in removing the muscle from the body. If such injury could be completely eliminated the resting value of tissue lactic acid would probably be even less than 15 mg. per cent. In the intact animal there will necessarily be present in tissues some lactic acid derived from the breaking down of lactacidogen, due to inevitable slight movements of muscles. Also, since lactic acid is freely diffusible into the blood, there will be a low, but fairly constant, amount of lactic acid in blood. Blood lactic acid is normally small in amount, values from 10 to 30 mg. per 100 cc. having been reported by various workers (Clausen, 1922; Long, 1924). After the injection of sodium lactate, therefore, the concentration of lactic acid in both blood and tissues is abnormally high, and this excessive amount of lactic acid must be disposed of by the body in some way.

Urine analyses after injection indicate that only negligible quantities of lactic acid were excreted. The results of two experiments are given in Table VII. In one experiment (Dog 3, second injection) the urine excreted within 1 hour after the injection became distinctly alkaline and showed effervescence on adding acid, indicating the presence of large amounts of bicarbonate. This suggested that some of the sodium which was combined with the sodium lactate is rapidly excreted as sodium bicarbonate, leaving behind the lactic acid. An approximate idea of the sodium bicarbonate excreted may be gained from the following calculations. In the absence of quantitative analytical data we may assume that after the injection of sodium lactate the reaction of the urine changed from pH 6.5 (acid to litmus) to pH 8 (alka-

line to litmus). Gamble (1922) has shown that the concentration of free carbonic acid in the urine remains practically constant, and that the concentration of bicarbonate is a linear function of pH. Urine at pH 6.5 would contain 0.46 mg. of bicarbonate per 100 cc., and at pH 8, 840 mg. per 100 cc. The total amount of bicarbonate excreted after the injection of sodium lactate in Dog

TABLE VII.

Reaction to litmus.	Urine volume.	Total lactic acid.	Excess lactic acid.*	Time.
Dog 3.				
	cc.	mg.	mg.	
	400	41.6		Jan. 17, 10 a.m.-5 p.m.
	110	10.8		" 17, 5 p.m.-Jan. 18, 10 a.m.
Acid.	11	1.3	0.2	Jan. 18, 10-11 a.m. At 10.25 a.m. sodium lactate equivalent to 1275 mg. lactic acid injected.
Alkaline.	12	5.0	3.7	Jan. 18, 11 a.m.-12 m.
"	15	2.6	1.0	" 18, 12 m.-5 p.m.
"	220	21.3	1.7	" 18, 5 p.m.-Jan. 19, 10 a.m.
Dog 4.				
	275	27.7		Jan. 10, 10 a.m.-5 p.m.
	50	8.3		" 10, 5 p.m.-Jan. 11, 10 a.m.
	60	12.7	4.6	Jan. 11, 10 a.m.-5 p.m. At 10.30 a.m. sodium lactate equivalent to 2125 mg. lactic acid injected.
	875	90.1	1.1	Jan. 11, 5 p.m.-Jan. 12, 10 a.m.

\* Amount of lactic acid in excess of the lactic acid excreted the previous day in an equal amount of urine.

3, second experiment, would be 0.005 gm. (11 cc. of urine at pH 6.5) plus 2.06 gm. (247 cc. of urine at pH 8), a total of 2.07 gm. In the same amount of urine at pH 6.5 there would be 0.118 gm. of bicarbonate. The difference, 1.86 gm., represents the excess bicarbonate excreted after the injection. As the equivalent of 1.5 gm. of sodium bicarbonate was injected the rough calculations made above suggest that all of the base injected is quickly excreted, although combined with only a trace of the lactic acid.



Some of the injected lactic acid is probably oxidized. Taistra (1921) found that lactic acid when fed causes an increase in heat production. However, Lusk (1921) found increased heat production was much less when sodium lactate was fed instead of lactic acid. In all cases, however, if the lactic acid given were completely burned the heat produced should have been much greater than that observed. This is in agreement with other forms of experimentation, where the lactic acid concentration reached a high level. Hill, working on the heat production in isolated muscle during contraction and recovery, found the heat produced accounted for the oxidation of only one-fifth of the lactic acid formed in contraction and removed during recovery in oxygen. Meyerhof (1920), also working on isolated muscle, showed that the oxygen consumed by the muscle was only sufficient to account for the removal by oxidation of one-third to one-fourth of the lactic acid formed in contraction. The work of Hill, Long, and Lupton (1924) on the effect of exercise on blood lactic acid in men, furnished further confirmation. These authors were able to show that for every liter of oxygen above the normal resting value consumed during recovery from exercise, 7 gm. of lactic acid were removed from the blood. This oxygen consumption again accounts for oxidation of only one-fifth of the lactic acid.

Hill, Long, and Lupton (1924, 1925) found that recovery, as indicated by increased oxygen intake, continued for 80 to 120 minutes after severe exercise. They had previously shown that the same exercise increased the blood lactic acid to 80 to 100 mg. per 100 cc. The removal of the lactic acid required a much longer time than the production. According to them part at least of the time required for recovery is due to the fact that a large quantity of lactic acid has diffused from the muscles into the blood and has been taken up by other tissues, which are unable to dispose of it. This means that the lactic acid in these other tissues must again be taken by the blood back to the muscles, where it is reconverted to glycogen. As the lactic acid in the blood is high, the return of lactic acid from these tissues to the blood and thence to muscle must await a time when blood lactic acid will be reduced to such a concentration as to allow diffusion of lactic acid from the tissues to the blood. Also the rate of removal of lactic acid in the muscles decreases as the concentration of lactic acid de-

creases. These two factors will tend to make the process of removal of lactic acid a slow one.

After the injection of sodium lactate into the blood, we have a condition in many ways comparable to conditions obtaining in the body after exercise. Lactic acid will diffuse from the blood to all tissues of the body, including the muscles, which are not high in lactic acid as they are after exercise. This diffusion at first will be rapid, due to the low concentration of lactic acid in the tissues. The rate of diffusion will decrease as the concentration of lactic acid in the blood approaches that in the tissues. After this point is reached diffusion from blood to muscles, and therefore from other tissues to blood, will depend upon the rate of removal in muscles.

Based on the work of Embden, Hill, and Meyerhof the general conception of the sequence of events in contraction and recovery of muscle is this: Glycogen combines with inorganic phosphate to form lactacidogen, which is broken down to lactic acid and inorganic phosphate. At the same time some glucose is formed from glycogen. In recovery this glucose is burned to supply the energy for the reversion of lactic acid and inorganic phosphate to lactacidogen, and ultimately to glycogen, with the liberation of phosphates. Removal of the injected lactic acid might also occur by a conversion of lactic acid to lactacidogen, and ultimately to glycogen, especially in view of the work of Meyerhof, Lohmann, and Meier (1925), who showed that an increase in glycogen content occurred in frog muscle when perfused with lactates. The energy for the conversion of the injected lactic acid to lactacidogen might come from oxidation of a small part of the lactic acid itself, or by oxidation of glucose already present in the blood, or glucose produced from glycogen for that purpose. Since phosphoric acid is also a part of the compound being synthesized, there should be utilization of body phosphates in synthesis of this compound. A study of the blood phosphates after injection might indicate that such is the case. The formation of lactacidogen occurring in the tissues, would first draw on tissue phosphates, but as tissue phosphates were decreased, phosphate would diffuse from blood to tissues. We might expect, therefore, that the greater the amount of phosphate needed to combine with the lactic acid the greater the change in blood phosphate would be. A consideration of the changes in blood phosphates

after injection of sodium lactate, as given in the above tables, shows that this is actually the case. We see that blood phosphate dropped 0.6 and 1.2 mg. when 850 mg. of lactic acid were injected; 1.5 mg. when 1275 mg. of lactic acid were given. In one experiment, after injection of 2125 mg. of lactic acid all the available inorganic phosphate of the blood was drawn upon, the concentration falling from 3.1 mg. to 0. The decrease in phosphate is not due to alkali injected. It was shown by Haldane, Wigglesworth, and Woodrow (1924) in experiments on ingestion of large amounts of sodium bicarbonate in man, that although in one experiment the urinary excretion of phosphate fell from 27 to 2.8 mg. per hour, this was not a constant phenomenon, and in no instance was there a significant change in blood phosphate. Fiske (1921) found that phosphate excretion was unchanged by ingestion of bicarbonate in amounts sufficient to render the urine alkaline. Indications are that phosphates are in some way concerned in the removal of the injected lactic acid. The most plausible explanation is that lactic acid and phosphate are combined to form lactacidogen.

There remains to be considered the increase in blood sugar found in the normal animals. This occurred definitely in three of the experiments, while in two there was an insignificant rise in sugar. In these five experiments the increase occurred always at a period when phosphates and lactic acid were returning to normal value. In Dog 5 the increase in sugar occurred immediately and was probably due to excitement. We might assume that the presence of abnormal amounts of lactic acid acts as a stimulus for the production of glucose to furnish energy for the removal of the lactic acid. When the stimulus for glucose production (high lactic acid) is removed, glycogen hydrolysis stops, and glucose in the blood, after reaching a certain maximum, will again gradually return to normal. Observations were not continued for a sufficiently long period in these experiments to show this return to normal.

The factor of alkali injection must also be considered here in relation to its effect on blood sugar. Haldane, Wigglesworth, and Woodrow found that ingestion of large amounts of sodium bicarbonate occasionally caused a rise in blood sugar of 20 per cent above normal, but that in many cases no definite rise was observed. No figures are given in their report. On the other

hand, Elias (1913) found acids increased blood sugar, and Kramer and Murlin (1915) report that injection of sodium carbonate causes a decreased blood sugar. Du Vigneaud and Karr (1925) found that sodium bicarbonate given during fasting increased the rate of disappearance of blood sugar in normal dogs during glucose tolerance test. It does not appear probable that the increase in sugar observed in the experiments reported here, even in the case of injection of the equivalent of 2.6 gm. of sodium bicarbonate (as sodium lactate) is due to the alkali, but is the result of a disturbance of that phase of carbohydrate metabolism involving lactic acid, phosphates, and sugar.

Haggard and Henderson (1920) report two experiments on injection of lactic acid. They found that the decrease in sodium bicarbonate after the injection of lactic acid into the blood was much smaller than that caused by injection of an equivalent amount of hydrochloric acid. The smaller effect of lactic acid on the alkaline reserve may be explained by a rapid conversion of lactic acid to glycogen, thus liberating again some of the base with which it had been combined. We may assume that synthesis of lactacidogen and glycogen begins immediately after the concentration of lactic acid becomes greater than normal. As Haggard and Henderson did not determine alkaline reserve until at least 15 minutes had elapsed it seems possible that synthesis of lactic acid to glycogen with liberation of base would explain their results.

The experiments on the diabetic dog are interesting in that they indicate the animal is able to deal with large amounts of lactic acid just as the normal animal does. Apparently there is no inability to form lactacidogen. Harrop and Benedict (1923) suggest that insulin accelerates the formation of a hexose diphosphate compound during storage of glycogen, but since removal of lactic acid with a coincident drop in phosphates occurs after injection of sodium lactate in this diabetic dog, insulin apparently is not necessary for lactacidogen formation. Bolliger and Hartman (1925) find injection of glucose into a completely depancreatized dog causes no drop in blood phosphates such as occurs in normal animals. They suggest that phosphates in the blood decrease only under the influence of insulin. This is also contradicted by the results of injection of sodium lactate into a diabetic dog.

## SUMMARY.

A. Sodium lactate injected into dogs in large amounts is readily removed from the blood. The removal may be divided into two phases:

1. A rapid decrease in concentration of lactic acid in the blood due to diffusion of lactic acid from the blood to other body fluids.

2. A slower decrease in concentration due to utilization of lactic acid by the tissues.

B. Injection of sodium lactate causes an immediate decrease in inorganic phosphate in the blood, and a delayed rise in the sugar of the blood.

C. The conclusion is drawn that lactic acid injected into the blood is synthesized to lactacidogen and glycogen by a process analogous to removal of lactic acid formed in muscle exercise.

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## MAGNESIUM CONTENT OF NORMAL RATS AT DIFFERENT AGES.

BY GRACE MEDES AND GERTRUDE J. HUMPHREY.

*(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)*

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In a preceding paper (1) a study of magnesium metabolism of albino rats was presented. A considerable part of the data represented determinations on normal rats which served as controls in the various experiments. But since the period studied covered only about 30 days of the growth period, it has seemed desirable to extend the work to include the entire period from birth to maturity.

While considerable work has been done upon the composition of the normal rat with respect to calcium and phosphorus, little has appeared in the literature on the magnesium content.

Buckner and Peter (2) prepared tables of the phosphorus, calcium, and magnesium content of rats from 2 to 40 weeks of age. According to their figures all three elements were on the average slightly greater in the female than in the male. The percentage of phosphorus and calcium increased with age during the entire period at about the same rate. On the other hand, the authors found no increase in percentage of magnesium. Their tables show a wide variability in magnesium content of different individuals, their determinations varying from 0.024 per cent (12 weeks) to 0.048 per cent (8 weeks).

Toverud (3) gives a table of inorganic constituents of new born rats in which he records magnesium in percentage of body weight. His determinations for two litters were 0.0206 and 0.0230 per cent respectively.

The rats used in the experiments here recorded were descended from a single litter. Their diet consisted of 65 per cent ground whole wheat, 32 per cent whole milk powder, 3 per cent cod liver oil, 1 per cent sodium chloride, and 1 per cent calcium carbonate. Their diet gave on analysis 0.69 per cent Ca, 0.44 per cent P, and 0.025 per cent Mg.

At the close of the experiment the rats were killed with chloroform, ashed in silica dishes, and the ash analyzed for calcium, magnesium, and phosphorus. The methods employed in these determinations have been described previously (1).

In Table I are recorded the average weights in gm. of rats at birth and at 30, 60, 90, 120, and 150 days respectively; their average compositions in respect to calcium, phosphorus, and magnesium; the percentage of body weight of each of these constituents, and the ratios of these three elements.

TABLE I.

*Showing Composition of Rats of Various Ages, with Respect to Ca, P, and Mg.*

No. of rats. Average.	Age.	Sex.	Average weight.	Composition.			Per cent of body weight.			Ratios.		
				Ca	P	Mg	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg
	days		gm.	mg.	mg.	mg.						
6	At birth.		4.6	12.2	15.4	2.14	0.26	0.34	0.048	1.31	6.3	6.6
4	30	♂	54	375	275	22	0.65	0.51	0.045	0.72	16.9	12.5
6	30	♀	45	314	251	19	0.72	0.58	0.039	0.80	13.5	13.5
5	60	♂	135	1078	774	62	0.80	0.58	0.046	0.75	17.39	12.87
5	60	♀	118	986	679	56	0.83	0.58	0.048	0.69	17.61	12.83
5	90	♂	218	1937	1297	95	0.89	0.60	0.041	0.67	20.39	12.98
5	90	♀	186	1819	1203	83	0.98	0.65	0.045	0.66	21.92	13.06
4	120	♂	281	2452	1618	96	0.87	0.58	0.034	0.66	25.54	16.85
3	120	♀	180	1830	1208	71	1.02	0.67	0.039	0.66	25.78	17.01
2	150	♀	172	1755	1123	71	1.03	0.66	0.041	0.64	24.72	15.82

Table I shows that there was an increase with age, in the absolute amount of magnesium, until 90 days, after which the number of mg. remained approximately constant. It was greater at any given age in the male than in the female. For any given weight, however, there was no sex difference.

The percentage of magnesium falls to a minimum at 30 days and rises to a maximum at 70 to 80 days. The fall is more marked in the female than in the male; accordingly, at 30 days the percentage of magnesium is greater in the male and after 60 days greater in the female. In comparing males and females of the same weight, females of 50 gm. weight have the lowest

percentage of magnesium, whereas after about 100 gm., the percentage of magnesium is greater in the female. At 180 gm., sex differences disappear.

The two female rats analyzed at 150 days were sister rats to the three females killed at 120 days. The two former had just produced litters while the three latter were virgin. Until the time of pregnancy, the rats in the two groups had weighed approximately the same. As may be seen from Table I, there was a loss of body weight during pregnancy, together with a decrease in absolute amount of calcium and phosphorus. The absolute amount of magnesium, however, remained constant. As a consequence, the percentage of magnesium was slightly raised. The percentage, however, did not deviate beyond the range of that of individual normal virgin females.

#### SUMMARY.

The absolute amount of magnesium in both males and females increased for 90 days, after which it remained about constant.

After 60 days body weight increased more rapidly than magnesium, so that the percentage of magnesium in the ash decreased.

Rats of any given weight showed much slighter sex differences in respect to magnesium content than rats of any given age.

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# THE ROTATORY DISPERSION OF THE PENTACETATES OF $\alpha$ - AND $\beta$ -GLUCOSE AND OF $\alpha$ - AND $\beta$ -MANNOSE.

BY P. A. LEVENE AND ISAAC BENCOWITZ.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

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The van't Hoff superposition theory of optical rotation has been applied with much success by Hudson for the explanation of the optical properties of isomeric mono- and polysaccharides. The method of Hudson proved to be of great service, not only for the analysis of the optical rotations of known forms, but also for the prediction of the rotations of forms not yet known. However, in certain instances, namely in the cases of mannose, lyxose, and rhamnose, the rules of Hudson do not hold. The reasons for the deviations are as yet unknown.

The optical rotation of sugars is generally measured in the light of sodium vapor or in light of the same wave-length, the most convenient solvents being employed. Often only one solvent is used, frequently with few variations in the concentrations of the solutions. On the other hand, it is known that all these variables—wave-length, solvent, and concentration—affect individual substances in an individual manner.

The effects produced on the rotations of the pentacetates of  $\alpha$ - and  $\beta$ -glucose and on the pentacetates of  $\alpha$ - and  $\beta$ -mannose by different solvents and different concentrations have been discussed in a previous publication.<sup>1</sup> In the present communication the values are given of the optical rotations of the same pentacetates measured at ten different wave-lengths for different concentrations. The rotatory dispersion of the pentacetate of  $\alpha$ -mannose has already been referred to in a previous communication.<sup>2</sup>

<sup>1</sup> Levene, P. A., and Bencowitz, I., *J. Biol. Chem.*, 1927, lxxiii, 679.

<sup>2</sup> Levene, P. A., and Bencowitz, I., *J. Biol. Chem.*, 1927, lxxii, 627.

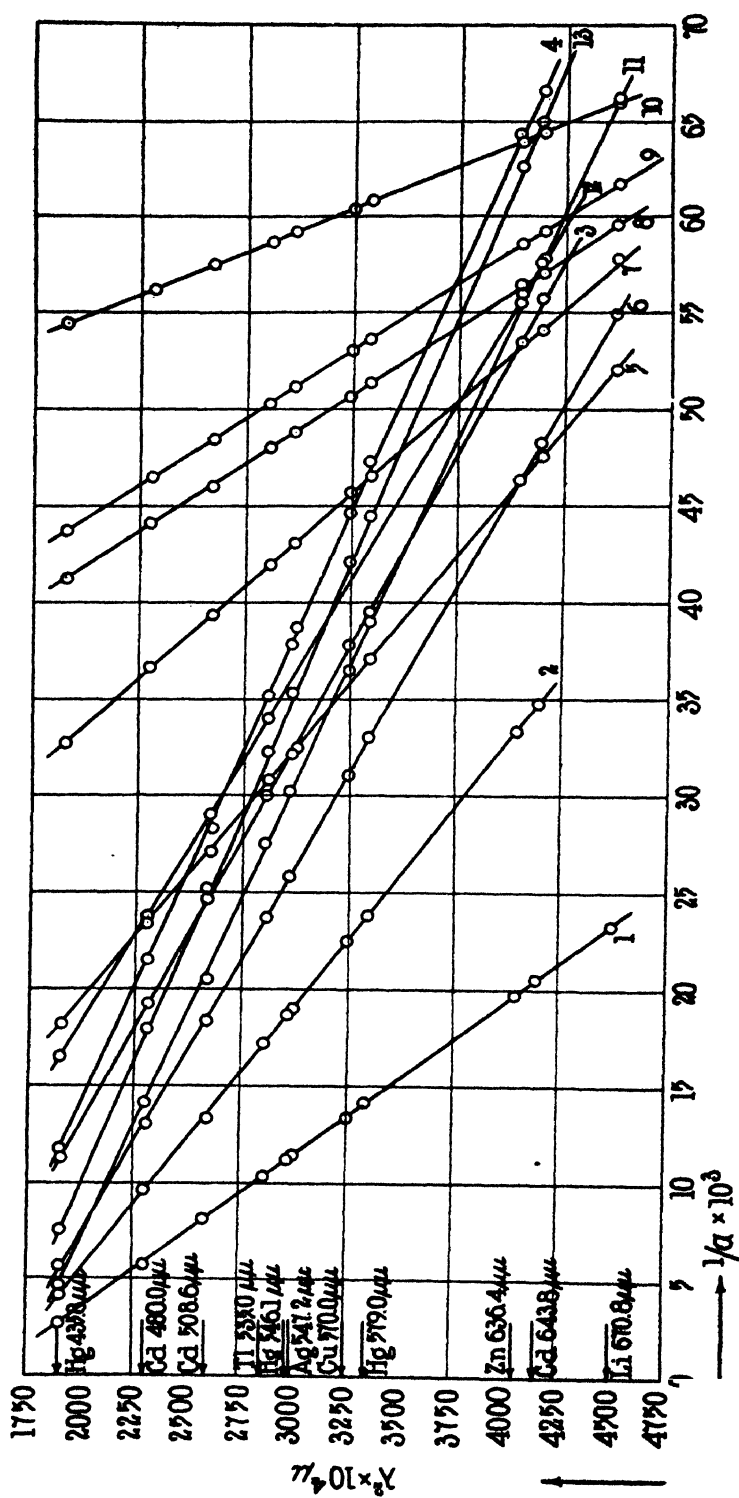


Fig. 1.



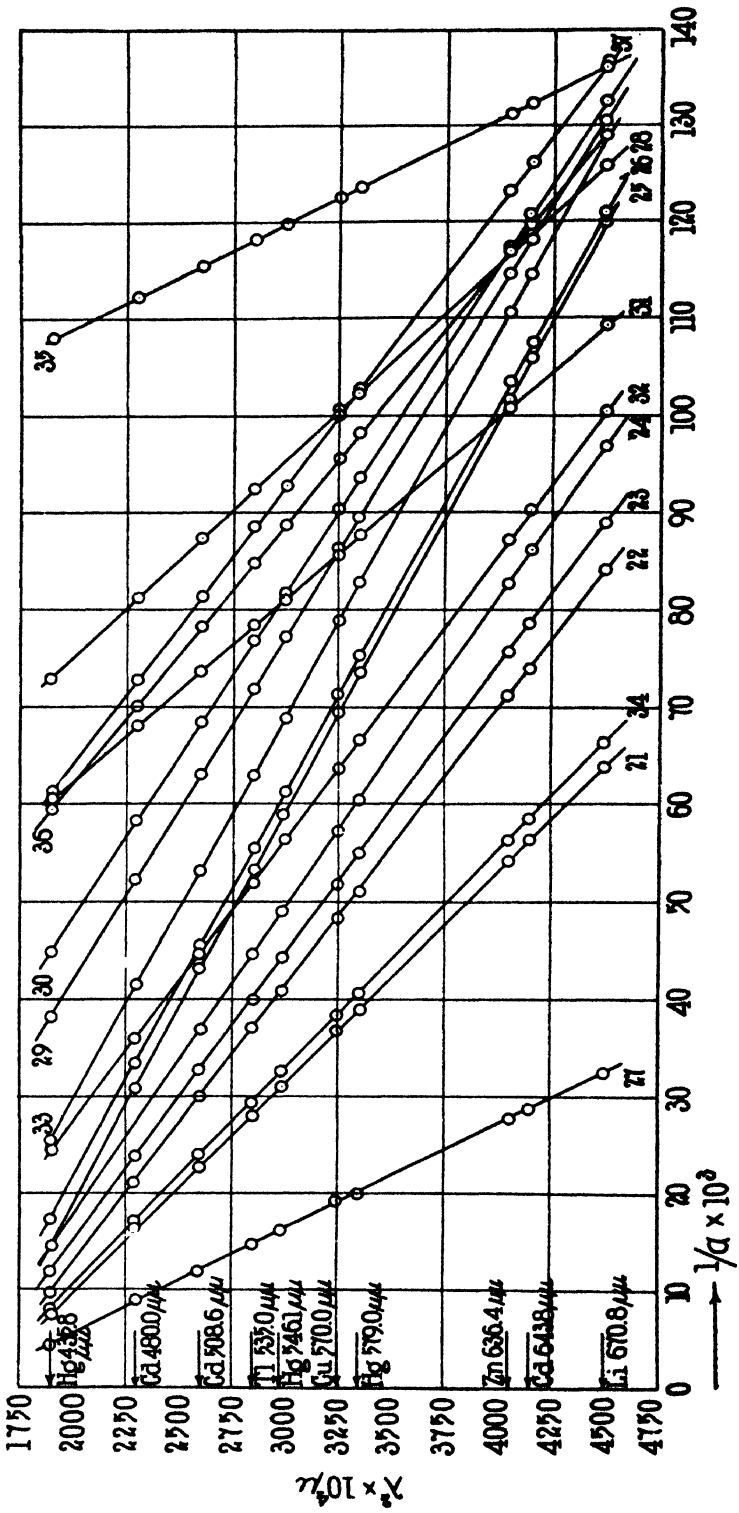


Fig. 2

Fig. 2.

21. $\beta$ -Mannose pentacetate in chloroform, concentration = 0.2870 gm. per cc.					
				Scale	$\alpha$ , subtract 25.0
22.	"	"	0.4287	"	" add 40.0.
23.	"	"	0.0211	"	" 15.0, multiply by 10.
24.	"	acetone	0.3476	"	" 40.0.
25.	"	"	0.1349	"	" 55.0.
26.	"	"	0.2779	"	" 55.0.
27. $\beta$ -Glucose	"	"	0.2380	"	" 10.0.
28. $\alpha$ -Glucose	"	chloroform	0.3100	"	divide by 2, subtract 2.0.
29.	"	acetone	0.1641	"	" 2, add 10.0.
30.	"	chloroform	0.0942	"	" 2, " 5.0.
31	"	acetone	0.3128	"	" 2, subtract 15.0.
32. $\beta$ -Galactose	"	"	0.1860	"	" 2, add 32.5.
33.	"	chloroform	0.1583	"	" 2, " 97.5.
34. $\alpha$ -Glucose	"	benzene	0.1462	"	" 2, " 14.5.
35. $\beta$ -Mannose	"	"	0.0950	"	subtract 90.0, multiply by 2.
36. $\beta$ -Glucose	"	chloroform	0.2870	"	" 25.0, " 10.
37.	"	benzene	0.0825	"	" 25.0, " 10.

As in the case previously reported, the experimental results presented here can be reproduced accurately by one term of Drude's equation:

$$[M] = \Sigma \frac{K}{\lambda^2 - \lambda_0^2}$$

This fact is shown in Figs. 1 and 2 in which the reciprocals of the experimental rotations, not modified by any factors, are plotted as abscissas and the squares of the wave-lengths (expressed in  $\mu$ ) as ordinates. The scales of the graphs were chosen so that the smallest division corresponded to a change in rotation of less than  $0.02^\circ$ . It is readily seen that with the exception of a few occasional measurements, all the points lie on straight lines.

A more striking proof that the simple equation is sufficient to express the experimental points within the range of Li red and Hg violet is given in Table I. In Columns 3, 4, 7, and 8 are given the observed and calculated molecular rotations. In Columns 5 and 9 are given the differences between the two values expressed in per cent. It is obvious from these data, not only from the slight magnitude of the errors but also from the irregularities of the directions of these differences, that the assumption of the simple equation is rigorous.

In Table III are given the values of  $K$  at round concentrations. These values are not necessarily identical with those given in Table I. The latter values are dependent upon the experimental concentrations and therefore their accuracy is limited by the accuracy of the concentrations. The molecular rotations interpolated from a smooth curve give the average values and the values of  $K$  obtained from these values are necessarily more accurate, or at least more consistent.

In Table IV are given the differences between the values of  $K$  for the  $\alpha$  and  $\beta$  forms of the pentacetate of glucose and of the pentacetate of mannose. Comparing the ratios

$$\frac{(K_\alpha - K_\beta) \text{ mannose pentacetate}}{(K_\alpha - K_\beta) \text{ glucose pentacetate}}$$

with the ratios

$$\frac{([M_\alpha] - [M_\beta]) \text{ mannose pentacetate}}{([M_\alpha] - [M_\beta]) \text{ glucose pentacetate}}$$

it is found that they have the same values. This agreement is to be expected when the course of the dispersion has the simple character capable of being expressed, as stated above, by a single term of Drude's equation.

The practical conclusion which follows from these observations is that for the solution of these problems in the case of the sugars which were studied by the optical method, the molecular rotations in any one monochromatic light are as significant as the dispersion constants.<sup>3</sup>

#### EXPERIMENTAL.

##### *Procedure.*

The apparatus employed, as well as the method used, was described in a recent publication.<sup>2</sup>

The green mercury line 5461, the violet 4359, and the yellow 5790, were obtained directly from a quartz mercury arc, the light from which was purified by means of a large, direct vision spectroscope. The rest of the lines used were obtained from a continuous spectrum, patches of which, having passed the spectroscope, were chosen so that the optical center of each patch had the desired wave-length. The optical centers were determined by means of a quartz test plate calibrated by the Bureau of Standards. The details of the procedure were described in an earlier publication.

##### *Calculations.*

The constant  $K$  was calculated in the usual way from Drude's formula. In Table III are given the values of this constant at round concentrations. The molecular rotations used for calculating the constants were taken from an earlier paper in which these values were read off from smooth curves drawn on large scales.

The constant  $\lambda_0^2$  is very sensitive to slight variations in  $\alpha$ , inasmuch as it is obtained by means of differences. The following modification of the simple equation was employed:

$$\lambda_0^2 = \frac{\alpha_1 \lambda_1^2 - \alpha_2 \lambda_2^2}{\alpha_1 - \alpha_2}$$

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<sup>3</sup> We are indebted to Dr. L. W. Bass for verifying the tables.



## Rotatory Dispersion

where  $\alpha$  is the experimental rotation as measured, not modified by any factors such as the concentrations or the lengths of the tubes.  $\alpha_1$  and  $\alpha_2$  were chosen so that the differences were as large as possible. As many combinations were employed as were found necessary to give each measurement equal weight. Often as many as twenty combinations were used.

The difference of  $\alpha$  for Hg 5461 and Hg 4358 suggests itself as the most reliable combination. However, with the more concentrated solutions the measurements with the Hg violet line were not as accurate as with the others. This fact was indicated by a larger average of the mean deviation from the average.

TABLE I.

*Comparison of Observed and Calculated Molecular Rotations for Different Wave-Lengths.*

$\alpha$ -Mannose pentacetate in chloroform. $\lambda_D^2 = 0.0315 \mu$ .								
Wave-length. $\mu$ (1)	Concentration = 0.0430 gm. per cc. $K = 6315$				Concentration = 0.0775 gm. per cc. $K = 6878$			
	$\alpha$ , 4 dm. tube.	[M] observed.	[M] calculated.	Difference	$\alpha$ , 2 dm. tube.	[M] observed.	[M] calculated.	Difference.
	(2) degrees	(3)	(4)	(5) per cent	(6) degrees	(7)	(8)	(9) per cent
0.6708								
0.6438	7.65	177.6	177.9	+0.16	7.12	179.1	179.6	+0.22
0.6364	7.87	182.7	182.5	-0.10	7.34	184.7	184.2	-0.21
0.5790	9.67	224.5	224.3	-0.08	9.00	226.4	226.4	0
0.5700	10.00	232.1	232.3	+0.08	9.34	235.0	234.4	-0.25
0.5472	10.97	255.0	254.4	-0.23	10.21	256.9	256.8	-0.03
0.5461	11.07	257.0	255.5	-0.58	10.25	257.7	257.9	+0.07
0.5351	11.52	267.0	267.6	+0.22	10.75	270.0	270.0	0
0.5086	12.91	299.0	299.9	+0.30	12.04	302.9	302.7	-0.06
0.4800	14.71	341.0	342.5	+0.44	13.76	346.2	345.7	-0.14
0.4359	15.51	429.0	429.9	+0.20	17.15	431.5	433.9	+0.32

Wave-length. $\mu$	Concentration = 0.1566 gm. per cc. $K = 6809$				Concentration = 0.2410 gm. per cc. $K = 6574$			
	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	degrees			per cent	degrees			per cent
0.6708								
0.6438	7.09	176.6	177.8	+0.67	10.63	172.0	171.7	-0.17
0.6364	7.31	182.1	182.3	+0.10	10.90	176.4	176.0	-0.22
0.5790	8.98	223.6	224.1	+0.22	13.37	216.4	216.4	0
0.5700	9.31	231.9	232.1	+0.08	13.82	223.6	224.1	+0.22
0.5472	10.20	254.0	254.1	+0.03	15.11	244.5	245.4	+0.36
0.5461	10.30	256.5	255.5	-0.46	15.30	247.6	246.5	-0.44
0.5351	10.76	268.0	267.3	-0.26	15.96	258.3	258.0	-0.11
0.5086	12.04	299.8	299.7	-0.03	17.90	289.7	289.4	-0.10
0.4800	13.78	343.2	342.3	-0.26	20.40	330.1	330.5	+0.12
0.4359	17.27	430.1	429.6	-0.11	25.52	413.0	414.8	+0.43

TABLE I.—*Continued.* $\alpha$ -Mannose pentacetate in chloroform.  $\lambda_0^2 = 0.0315 \mu$ .

Wave-length. $\mu$	Concentration = 0.3095 gm. per cc. $K = 6413$				Concentration = 0.4580 gm. per cc. $K = 6251$			
	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	<i>degrees</i>			<i>per cent</i>	<i>degrees</i>			<i>per cent</i>
0.6708								
0.6438	13.22	166.6	167.4	+0.47	19.19	163.4	163.2	-0.12
0.6364	13.61	171.5	171.7	+0.11	19.67	167.5	167.4	-0.06
0.5790	16.81	211.8	211.1	-0.33	24.16	205.7	205.8	+0.04
0.5760	17.31	218.1	218.6	+0.22	24.97	212.6	213.0	+0.18
0.5472	19.05	240.0	239.4	-0.25	27.44	233.6	233.3	-0.12
0.5461	19.14	241.2	240.5	-0.29	27.60	235.0	234.4	-0.25
0.5351	19.97	251.6	251.7	+0.04	28.86	245.7	245.3	-0.16
0.5086	22.40	282.3	282.3	0	32.36	275.5	275.1	-0.15
0.4800	25.55	322.0	322.4	+0.12	36.85	313.8	314.3	+0.15
0.4359	32.01	403.3	404.6	+0.32	46.08	392.4	394.4	+0.51

Wave-length. $\mu$	Concentration = 0.8033 gm. per cc. $K = 6090$			
	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	<i>degrees</i>			<i>per cent</i>
0.6708				
0.6438	32.79	158.8	159.0	+0.12
0.6364	33.61	163.2	163.1	-0.06
0.5790	41.33	200.7	200.5	-0.09
0.5700	42.74	207.5	207.6	+0.04
0.5472	46.93	227.8	227.3	-0.22
0.5461	47.16	229.0	228.4	-0.26
0.5351	49.27	239.2	239.0	-0.09
0.5086	55.26	268.2	268.1	-0.03
0.4800	62.66	304.1	306.1	+0.65
0.4359	79.18	384.4	384.2	-0.06

TABLE I—Continued.

 $\alpha$ -Mannose pentacetate in benzene.  $\lambda_D^2 = 0.0326 \mu$ .Concentration = 0.1051 gm. per cc.  $K = 7277$ 

$\mu$	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	degrees			per cent
0.6708				
0.6438	5.13	190.2	190.5	+0.09
0.6364	5.26	195.3	195.4	+0.04
0.5790	6.49	241.1	240.4	-0.25
0.5700	6.70	248.8	249.0	+0.03
0.5472				
0.5461	7.37	274.0	274.0	0
0.5351	7.72	286.7	286.8	+0.03
0.5086	8.66	321.8	321.8	0
0.4800	8.90	367.7	367.9	+0.04
0.4359	12.45	462.2	462.3	+0.01

 $\alpha$ -Mannose pentacetate in acetone.  $\lambda_D^2 = 0.0306 \mu$ .Concentration = 0.02306 gm. per cc.  
 $K = 6046$ Concentration = 0.0352 gm. per cc.  
 $K = 6288$ 

Wave-length.	$\alpha$ 4 dm. tube.	[M] observed.	[M] calculated.	Difference.		$\alpha$ 4 dm. tube.	[M] observed.	[M] calculated.	Difference.
	degrees			per cent		degrees			per cent
0.6708	3.40	143.8	144.2	+0.28		5.24	150.1	149.9	-0.13
0.6438	3.74	157.8	157.5	-0.19		5.91	163.7	163.8	+0.06
0.6364	3.83	161.9	161.5	-0.27		6.07	168.1	168.0	-0.06
0.5790	4.72	199.4	198.4	-0.50		7.46	206.6	206.4	-0.09
0.5700	4.86	205.4	205.4	0		7.72	213.8	213.7	-0.04
0.5472									
0.5461	5.34	225.7	225.9	+0.09		8.48	234.9	235.0	+0.04
0.5351	5.58	235.9	236.4	+0.22		8.87	245.7	245.9	+0.08
0.5086	6.26	264.0	265.1	+0.43		9.94	275.0	275.6	+0.22
0.4800	7.12	301.0	302.6	+0.53		11.31	313.0	314.7	+0.54
0.4359	8.93	378.0	379.3	+0.34		14.24	394.0	394.5	+0.12

TABLE I—*Continued.* $\alpha$ -Mannose pentacetate in acetone.  $\lambda_D^2 = 0.0306 \mu$ .

Wave-length. $\mu$	Concentration = 0.0744 gm. per cc. $K = 6287$				Concentration = 0.2249 gm. per cc. $K = 6289$			
	$\alpha$ 4 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 2 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708					17.22	149.1	149.9	+0.53
0.6438	12.11	163.5	163.7	+0.12	18.83	163.3	163.8	+0.31
0.6364	12.44	167.6	167.9	+0.18	19.30	167.3	168.0	+0.41
0.5790	15.31	206.7	206.4	-0.14	23.77	206.0	206.4	+0.19
0.5700	15.83	213.8	213.5	-0.14	24.60	213.3	213.7	+0.18
0.5472	17.34	234.1	233.9	-0.08				
0.5461	17.40	234.9	234.9	0	27.10	235.0	235.0	0
0.5351	18.25	246.4	245.9	-0.20	28.38	246.1	246.0	-0.04
0.5086	20.45	276.0	275.6	-0.10	31.75	275.2	275.7	+0.18
0.4800	23.30	314.0	314.7	+0.21	36.56	317.0	314.8	-0.68
0.4359	29.05	392.0	394.4	+0.60	45.13	390.2	394.5	+1.00

Wave-length. $\mu$	Concentration = 0.2674 gm. per cc. $K = 6263$				Concentration = 0.3329 gm. per cc. $K = 6148$			
	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708	10.11	148.5	149.3	+0.53	12.50	146.4	146.6	+0.13
0.6438	11.08	162.6	163.1	+0.30	13.66	160.0	160.1	+0.06
0.6364	11.36	166.7	167.3	+0.35	14.03	164.4	164.2	-0.12
0.5790	14.01	205.6	205.5	-0.04	17.25	202.1	201.8	-0.14
0.5700	14.50	212.8	212.8	0	17.84	209.0	208.9	-0.05
0.5472					19.54	228.9	228.7	-0.08
0.5461	15.96	234.3	234.0	-0.12	19.64	230.1	229.7	-0.17
0.5351	16.67	244.7	244.9	+0.08	20.55	240.7	240.5	-0.08
0.5086	18.87	277.0	274.6	-0.87	23.03	269.8	269.5	-0.10
0.4800	21.46	315.0	313.4	-0.51	26.27	307.8	307.7	-0.03
0.4359	26.68	391.6	392.9	+0.33	32.70	383.1	385.7	+0.67

TABLE I—Continued.

 $\alpha$ -Mannose pentacetate in acetone.  $\lambda_0^2 = 0.0306 \mu$ .

Wave-length. $\mu$	Concentration = 0.4998 gm. per cc. $K = 605$				Concentration = 0.6871 gm. per cc. $K = 5915$			
	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708	18.35	143.2	143.2	0	24.80	140.8	141.0	+0.15
0.6438	19.99	156.0	156.4	+0.25	27.15	154.1	154.1	0
0.6364	20.53	160.2	160.4	+0.12	27.80	157.8	158.0	+0.12
0.5790	25.26	197.5	197.1	-0.20	34.31	194.7	194.1	-0.30
0.5700	26.15	204.0	204.0	0	35.41	201.0	201.0	0
0.5472								
0.5461	28.76	224.4	224.4	0	38.99	221.2	221.0	-0.09
0.5351	30.12	235.0	234.8	-0.08	40.75	231.3	231.3	0
0.5086	33.77	263.5	263.3	-0.08	45.67	259.2	259.3	+0.04
0.4800	38.56	300.9	300.6	-0.10	52.10	295.7	296.0	+0.01
0.4359	48.40	377.7	376.7	-0.21				

 $\beta$ -Mannose pentacetate in acetone.  $\lambda_0^2 = 0.0185\mu$ .

Wave length. $\mu$	Concentration = 0.1349 gm. per cc. $K = 3571$				Concentration = 0.2779 gm. per cc. $K = 3548$			
	$\alpha$ 2 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708	-5.72	-82.70	-82.76	+0.07	-5.84	-82.01	-82.22	+0.25
0.6438	-6.22	-89.90	-90.18	+0.31	-6.36	-89.25	-89.59	+0.37
0.6364	-6.39	-92.4	-92.40	0	-6.52	-91.50	-91.80	+0.32
0.5790	-7.81	-113.0	-112.7	-0.23	-7.99	-112.1	-112.0	-0.08
0.5700	-8.05	-116.4	-116.5	+0.08	-8.25	-115.8	-115.8	0
0.5472								
0.5461	-8.83	-127.6	-127.6	0	-9.05	-126.9	-126.8	-0.08
0.5351	-9.22	-133.3	-133.3	0	-9.45	-132.6	-132.5	-0.07
0.5086	-10.31	-149.0	-148.7	-0.20	-10.55	-148.0	-147.7	-0.20
0.4800	-11.65	-168.4	-168.5	+0.06	-11.94	-167.6	-167.4	-0.11
0.4359					-14.80	-207.7	-206.9	-0.38

TABLE I—Continued.

 $\beta$ -Mannose pentacetate in acetone.  $\lambda_0^2 = 0.0185 \mu$ .Concentration = 0.3476 gm. per cc.  $K = 3531$ 

$\mu$	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	<i>degrees</i>			<i>per cent</i>
0.6708	−7.30	−82.0	−81.8	−0.24
0.6438	−7.95	−89.4	−89.2	−0.22
0.6364	−8.14	−91.5	−91.4	−0.12
0.5790	−9.93	−111.6	−111.5	−0.08
0.5700	−10.24	−115.1	−115.2	+0.08
0.5472				
0.5461	−11.22	−126.1	−126.1	0
0.5351	−11.73	−131.8	−131.8	0
0.5086	−13.06	−146.8	−147.0	+0.13
0.4800	−14.79	−166.3	−166.6	+0.18
0.4359	−18.28	−205.5	−205.9	+0.19

 $\beta$ -Mannose pentacetate in benzene.  $\lambda_0^1 = 0.0239 \mu$ .Concentration = 0.0950 gm. per cc.  $K = 3797$ 

Wave-length. $\mu$	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	<i>degrees</i>			<i>per cent</i>
0.6708	−2.17	−89.05	−89.11	+0.06
0.6438	−2.29	−97.05	−97.21	+0.16
0.6364	−2.43	−99.59	−99.63	+0.03
0.5790	−2.98	−122.2	−121.9	−0.24
0.5700	−3.07	−125.9	−126.1	+0.15
0.5472				
0.5461	−3.52	−138.3	−138.4	+0.07
0.5351	−3.94	−144.6	−144.7	+0.06
0.5086	−4.48	−161.6	−161.7	+0.06
0.4800	−5.58	−184.1	−183.9	−0.10
0.4359	−6.94	−229.1	−228.6	−0.22

TABLE I—Continued.

 $\beta$ -Mannose pentacetate in chloroform.  $\lambda_D^{25} = 0.0159\mu$ .

Wave-length. $\mu$	Concentration = 0.0211 gm. per cc. $K = 3106$				Concentration = 0.2870 gm. per cc. $K = 3142$			
	$\alpha$ 4 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 2 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708	-1.55	-71.60	-71.57	-0.04	-10.65	-72.30	-72.40	+0.13
0.6438	-1.69	-78.00	-77.94	-0.07	-11.58	-78.70	-78.85	+0.19
0.6364	-1.73	-79.90	-79.85	-0.06	-11.87	-80.60	-80.77	+0.21
0.5790	-2.11	-97.40	-97.28	-0.12	-14.49	-98.40	-98.40	0
0.5700	-2.17	-100.2	-100.5	+0.29	-14.99	-101.8	-101.7	-0.09
0.5472								
0.5461	-2.37	-109.4	-110.1	+0.63	-16.38	-111.3	-111.3	0
0.5351	-2.49	-115.0	-114.9	-0.08	-17.11	-116.2	-116.2	0
0.5086	-2.77	-127.9	-128.0	+0.07	-19.09	-129.7	-129.5	-0.15
0.4800	-3.15	-145.4	-144.9	-0.35	-21.54	-146.3	-146.5	+0.13
0.4359	-3.86	-178.2	-178.5	+0.16	-26.63	-180.9	-180.6	-0.16

Wave-Length. $\mu$	Concentration = 0.4287 gm. per cc. $K = 3178$			
	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	degrees			per cent
0.6708	-8.05	-73.23	-73.23	0
0.6438	-8.77	-79.78	-79.75	-0.03
0.6364	-8.97	-81.60	-81.70	+0.12
0.5790	-10.95	-99.61	-99.53	-0.08
0.5700	-11.33	-103.1	-102.9	-0.19
0.5472				
0.5461	-12.35	-112.4	-112.6	+0.17
0.5351	-12.94	-117.7	-117.6	-0.08
0.5086	-14.40	-131.0	-131.0	0
0.4800	-16.29	-148.2	-148.2	0
0.4359	-20.06	-182.5	-182.6	+0.06



TABLE I—Continued.

 $\alpha$ -Glucose pentacetate in benzene.  $\lambda_0^2 = 0.0258\mu$ .Concentration = 0.1462 gm. per cc.  $K = 11880$ 

Wave-length. $\mu$	$\alpha$ 2 dm. tube.	[M] observed.	[M] calculated.	Difference.
	<i>degrees</i>			<i>per cent</i>
0.6708	20.99	140.0	140.0	0
0.6438	22.96	152.8	152.8	0
0.6364	23.50	156.7	156.6	-0.03
0.5790	28.86	192.4	191.9	-0.25
0.5700	29.76	198.4	198.7	+0.12
0.5472				
0.5461	32.68	217.9	218.0	+0.05
0.5351	34.15	227.7	228.0	+0.10
0.5086	38.24	255.0	255.0	0
0.4800	43.48	290.0	290.3	+0.10
0.4359	54.27	362.0	361.8	-0.06

 $\alpha$ -Glucose pentacetate in chloroform.  $\lambda_0^2 = 0.0260\mu$ .

Wave-length. $\mu$	Concentration = 0.0942 gm. per cc. $K = 12360$				Concentration = 0.3100 gm. per cc. $K = 12430$			
	$\alpha$ 2 dm. tube.	[M] observed.	[M] calculated.	Difference.	$\alpha$ 1 dm. tube.	[M] observed.	[M] calculated.	Difference.
	<i>degrees</i>			<i>per cent</i>	<i>degrees</i>			<i>per cent</i>
0.6708	14.08	291.2	291.5	+0.10	23.24	292.4	293.1	+0.20
0.6438	15.33	317.3	318.1	+0.22	25.38	319.3	319.9	+0.18
0.6364	15.75	326.0	326.1	+0.03	26.06	327.8	328.0	+0.06
0.5790	19.31	399.7	399.6	-0.02	31.91	401.4	401.9	+0.12
0.5700	19.96	413.2	413.5	+0.07	33.05	415.8	415.9	+0.02
0.5472								
0.5461	21.98	450.0	454.1	+0.90	36.35	457.4	456.6	-0.17
0.5351	22.97	475.5	474.8	-0.14	37.99	477.9	477.5	-0.08
0.5086	25.69	532.0	531.2	-0.15	42.52	534.9	534.2	-0.11
0.4800	29.17	604.0	604.7	+0.11	48.36	608.4	608.1	-0.04
0.4359	36.24	750.0	753.7	+0.49	60.21	757.5	757.9	+0.05

TABLE I—Continued.

 $\alpha$ -Glucose pentacetate in acetone.  $\lambda_0^2 = 0.0265\mu$ .

Wave-length. $\mu$	Concentration = 0.1641 gm. per cc. $K = 13360$				Concentration = 0.3128 gm. per cc. $K = 13320$			
	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708	13.27	315.4	315.5	+0.03	25.16	313.7	314.5	+0.25
0.6438	14.48	344.1	344.3	+0.04	27.53	343.3	343.3	0
0.6364	14.89	353.9	353.0	-0.25	28.23	352.0	351.9	-0.03
0.5790	18.20	432.5	432.6	+0.02	34.60	431.4	431.3	-0.02
0.5700	18.83	447.5	447.7	+0.04	35.83	446.7	446.4	-0.07
0.5472								
0.5461	20.68	491.5	491.7	+0.04	39.34	490.5	490.3	-0.04
0.5351	21.66	514.8	514.2	-0.11	41.18	513.4	512.7	-0.11
0.5086	24.22	575.6	575.4	-0.03	46.07	574.4	573.6	-0.14
0.4800	27.60	655.9	655.2	-0.10	52.44	653.8	653.3	-0.08
0.4359	34.30	815.2	817.1	+0.23	65.27	813.8	814.8	+0.12

 $\alpha$ -Glucose pentacetate in pyridine.  $\lambda_0^2 = 0.0231\mu$ .

Wave-length. $\mu$	Concentration = 0.1998 gm. per cc. $K = 8689$				Concentration = 0.3604 gm. per cc. $K = 8890$			
	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708	10.41	203.2	203.5	+0.14	15.98	207.5	208.2	+0.33
0.6438	11.33	221.2	222.0	+0.36	17.45	226.6	227.1	+0.22
0.6364	11.65	227.4	227.5	+0.04	17.87	234.7	232.8	-0.81
0.5790	14.26	278.4	278.3	-0.04	21.93	284.7	284.8	+0.04
0.5700	14.74	287.8	287.9	+0.03	22.66	294.2	294.6	+0.13
0.5472								
0.5461	16.18	315.9	316.0	+0.03	24.89	323.2	323.3	+0.03
0.5351	16.91	330.2	330.1	-0.03	26.02	337.9	337.8	-0.02
0.5086	18.90	368.9	368.8	-0.02	29.13	378.2	377.3	-0.24
0.4800	21.50	419.7	419.2	-0.12	32.96	428.0	428.8	+0.18
0.4359	26.72	521.6	520.6	-0.18	40.98	532.1	532.6	+0.09

TABLE I—Continued.

 $\beta$ -Glucose pentacetate.In benzene.  $\lambda_D^2 = 0.0663\mu$ .In chloroform.  $\lambda_D^2 = 0.0595\mu$ .

Wave-length. $\mu$	Concentration = 0.0825 gm. per cc. $K = 405.7$				Concentration = 0.2870 gm. per cc. $K = 511.8$			
	$\alpha$ 4 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 1 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	degrees			per cent	degrees			per cent
0.6708	0.89	10.57	10.57	0	0.96	13.10	13.11	+0.07
0.6438	0.98	11.64	11.65	+0.08	1.06	14.41	14.41	0
0.6364	1.01	11.97	11.98	+0.08	1.09	14.80	14.81	+0.06
0.5790	1.28	15.13	15.08	-0.33	1.37	18.60	18.56	-0.21
0.5700	1.33	15.69	15.69	0	1.42	19.27	19.28	+0.05
0.5472								
0.5461	1.48	17.50	17.50	0	1.58	21.43	21.44	+0.04
0.5351	1.56	18.44	18.44	0	1.66	22.57	22.57	0
0.5086	1.78	21.09	21.09	0	1.89	25.69	25.69	0
0.4800	2.09	24.67	24.72	+0.20	2.20	29.93	29.95	+0.06
0.4359	2.77	32.75	32.80	+0.15	2.89	39.22	39.22	0

 $\beta$ -Glucose pentacetate in acetone.  $\lambda_D^2 = 0.0569\mu$ .

Wave-length. $\mu$	Concentration = 0.2363 gm. per cc. $K = 761.7$			
	$\alpha$ 2 dm. tube.	[M] observed.	[M] calculated.	Difference.
	degrees			per cent
0.6708	2.34	19.38	19.38	0
0.6438	2.58	21.31	21.30	-0.04
0.6364	2.65	21.89	21.88	-0.04
0.5790	3.35	27.38	27.36	-0.09
0.5700	3.43	28.34	28.42	+0.28
0.5472				
0.5461	3.82	31.57	31.70	+0.41
0.5351	4.02	33.21	33.20	-0.03
0.5086	4.57	37.77	37.75	-0.05
0.4800	5.32	43.92	43.90	-0.04
0.4359	6.94	57.30	57.23	-0.12

TABLE I—*Concluded.* $\beta$ -Galactose pentacetate.In chloroform.  $\lambda_0^1 = 0.0384\mu$ .In acetone.  $\lambda_0^2 = 0.0365\mu$ .

Wave-length. $\mu$	Concentration = 0.1583 gm. per cc. $K = 3102$				Concentration = 0.1860 gm. per cc. $K = 3609$			
	$\alpha$ 2 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.	$\alpha$ 2 dm. tube.	[M] ob- served.	[M] cal- culated.	Differ- ence.
	<i>degrees</i>			<i>per cent</i>	<i>degrees</i>			<i>per cent</i>
0.6708	6.10	75.1	75.3	+0.26	8.29	86.9	87.2	+0.34
0.6438	6.69	82.4	82.5	+0.12	9.08	95.2	96.1	+0.93
0.6364	6.86	84.5	84.6	+0.11	9.93	97.8	97.9	+0.10
0.5790	8.49	104.5	104.5	0	11.52	120.7	120.7	0
0.5700	8.81	108.5	108.3	-0.18	11.96	125.4	125.1	-0.23
0.5472								
0.5461	9.70	119.4	119.4	0	13.11	137.4	137.9	+0.36
0.5351	10.15	125.0	125.2	+0.15	13.82	144.9	144.4	-0.34
0.5086	11.46	141.1	140.8	-0.21	15.55	163.0	162.4	-0.36
0.4800	13.17	162.2	161.6	-0.37	17.85	187.2	186.1	-0.57
0.4359	16.57	204.1	204.6	+0.24	22.36	234.4	235.1	+0.29

TABLE II.  
*Values of the Constants  $\lambda_0^2$  and  $K$  in Table I.*

Substance.	Solvent.	Concentration in gm. per cc. $\times 10^3$ .	$\lambda_0^2$	$K \times 10^{-1}$
$\alpha$ -Mannose pent- acetate.	Chloroform.	4.300	$\mu$ 0.0315	63.15
		7.750		68.78
		15.66		68.09
		24.10		65.74
		30.95		64.13
		45.80		62.51
		80.33		60.90
		10.51	0.0326	72.77
	Benzene. Acetone.	2.306	0.0306	60.46
		3.520		62.88
		7.440		62.87
		22.49		62.89
		26.74		62.63
		33.29		61.48
		49.98		60.05
		68.71		59.15
$\beta$ -Mannose pent- acetate.	Chloroform.	2.110	0.0159	31.06
		28.70		31.42
		42.87		31.78
	Acetone.	13.49	0.0185	35.71
		27.79		35.48
		34.76		35.31
$\alpha$ -Glucose pent- acetate.	Benzene.	9.500	0.0239	37.97
	Chloroform.	9.420	0.0260	123.6
		31.00		124.3
	Acetone.	16.41	0.0265	133.6
		31.28		133.2
	Benzene.	14.62	0.0258	118.8
	Pyridine.	19.98	0.0231	86.89
		30.04		88.90
$\beta$ -Glucose pent- acetate.	Chloroform.	28.70	0.0595	5.118
	Acetone.	23.60	0.0569	7.617
	Benzene.	8.250	0.0663	4.057
$\beta$ -Galactose pent- acetate.	Chloroform.	15.83	0.0384	31.02
	Acetone.	18.60	0.0365	36.09

TABLE III.  
*Values of K at Round Concentrations.*  
 $K \times 10^{-2}$

Concentration in gm. per cc. $\times 10^3$ .	$\alpha$ -Mannose pentacetate.			$\beta$ -Mannose pentacetate.		
	Chloroform.	Acetone.	Benzene.	Chloroform.	Acetone.	Benzene.
2.00	66.67	58.34	72.51	31.04	34.99	38.40
4.00	65.07	61.01	72.77	31.04	35.17	38.40
6.00	67.47	62.08	72.77	31.04	35.41	38.57
8.00	67.74	62.35	72.77	31.04	35.69	38.74
10.0	67.74	62.62	73.31	31.04	35.83	37.58
12.0	68.01	62.62	72.51	31.04	35.83	37.30
14.0	68.01	62.62	72.77	31.04	35.83	36.61
16.0	68.11	62.62	72.51	31.04	35.83	36.94
18.0	67.55	62.75	72.06	31.04	35.83	36.21
20.0	67.34	62.78	72.24	31.04	35.83	36.21
22.0	66.56	62.75	71.71	31.04	35.83	36.12
24.0	65.93	62.78	71.31	31.32	35.55	35.93
25.0	65.39	62.78	71.02	31.32	35.55	35.84
30.0	64.57	62.06	70.23	31.32	35.55	35.66
35.0	63.77	61.36		31.61	35.27	
40.0	63.07	60.82		31.61	35.27	
45.0	64.19	60.42		31.61	35.27	
50.0	62.33	60.08		31.61		
60.0	61.79	59.51		31.61		
70.0	61.39					
80.0	60.97					

Concentration in gm. per cc. $\times 10^3$ .	$\alpha$ -Glucose pentacetate.			$\beta$ -Glucose pentacetate.		
	Chloroform.	Acetone.	Benzene.	Chloroform.	Acetone.	Benzene.
2.00	122.1	132.5	118.4	5.59	7.02	4.29
4.00	123.9	132.5	118.4	5.23	7.06	4.29
6.00	124.4	132.5	118.4	5.16	7.02	4.31
8.00	124.3	132.8	118.7	5.04	7.05	4.34
10.0	123.9	132.9	118.5	5.04	7.02	4.34
12.0	123.9	133.0	118.4	5.01	7.02	4.34
14.0	123.6	133.1	118.4	4.96	7.16	4.29
16.0	123.9	133.4	118.7	4.96	7.28	
18.0	124.1	133.3	119.5	4.96	7.40	
20.0	124.2	133.2	120.0	4.94	7.80	
22.0	124.5	133.1	120.4	4.92	7.57	
24.0	124.7	133.1	120.7	4.92	7.58	
26.0	124.9	133.2	120.9	4.96	7.64	
28.0	125.1	133.1	121.0	5.06	7.68	
30.0	125.0	133.0		5.25	7.71	
35.0	125.6	133.0		5.37		
39.0	126.0	133.0		5.44		
44.0	126.2			5.61		

TABLE IV.

*Differences in Values of K for  $\alpha$ - and  $\beta$ -Pentacetates at the Same Concentration.*

Concentration in gm. per cc. $\times 10^3$	Mannose pentacetate. $K_\alpha - K_\beta$			Glucose pentacetate. $K_\alpha - K_\beta$		
	Chloroform.	Acetone.	Benzene.	Chloroform.	Acetone.	Benzene.
2.00	97.71	93.33	110.91	115.5	125.5	114.1
4.00	96.11	96.18	111.17	118.7	125.4	114.1
6.00	98.51	97.49	111.34	119.3	125.5	114.1
8.00	98.78	98.04	111.51	119.3	125.8	114.4
10.0	98.78	98.45	110.89	118.9	125.9	114.2
12.0	99.05	98.45	109.81	118.9	126.0	114.1
14.0	99.05	98.45	109.38	118.6	126.0	114.1
16.0	99.15	98.45	109.45	118.9	126.1	
18.0	98.59	98.57	108.27	119.1	125.9	
20.0	98.38	98.61	108.45	119.2	125.7	
22.0	97.60	98.58	107.83	119.5	125.5	
24.0	97.25	98.33	107.24	119.7	125.5	
25.0	96.71	98.33	106.86	119.9	125.6	
30.0	95.89	97.61	105.89	119.8	125.3	
35.0	95.35	96.63		120.3		
40.0	94.68	96.09		120.6		
45.0	95.80	95.69		120.6		
50.0	93.94					
60.0	93.40					
70.0						
80.0						

## COZYMASE. A STUDY OF PURIFICATION METHODS.\*

By ALBERT L. RAYMOND† AND HOWARD M. WINEGARDEN.

(From the Gates Chemical Laboratory, California Institute of Technology, Pasadena.)

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Cozymase is one of the essential components of the complex enzyme mixture which effects alcoholic fermentation in the absence of living cells. The separation of the mixture into zymase and cozymase was first accomplished by Harden and Young<sup>1</sup> by means of ultrafiltration through a gelatin-impregnated Chamberland filter candle. The residue and filtrate as thus prepared possessed, separately, no fermentative action, but when mixed were found to produce a rapid fermentation. The active constituent of the residue was named zymase, while that constituent of the filtrate responsible for the reactivation of the residue was named cozymase.

The mechanism of the activation of the zymase was investigated by von Euler and Myrbäck,<sup>2</sup> who came to the conclusion that the cozymase was involved in that stage of the process in which inorganic phosphates are converted to carbohydrate esters. The same authors<sup>3</sup> have described a comprehensive and successful series of experiments on the purification of cozymase.

A complete knowledge of the properties and function of cozymase would be of extreme importance, as it should throw considerable light upon the mechanism of enzyme action as a whole, and should in addition have important bearing upon the entire question of carbohydrate utilization. It has been shown, for ex-

\* Contribution No. 122 from the Gates Chemical Laboratory, California Institute of Technology, Pasadena.

† National Research Fellow in Chemistry.

<sup>1</sup> Harden, A., and Young, W. J., *J. Physiol.*, 1905, xxxii, p. i; *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 405.

<sup>2</sup> von Euler, H., and Myrbäck, K., *Z. physiol. Chem.*, 1924, cxxxix, 15.

<sup>3</sup> von Euler, H., and Myrbäck, K., *Z. physiol. Chem.*, 1924, cxxxix, 281.



ample, by Meyerhof<sup>4</sup> that a substance, either identical with, or very similar to cozymase exists in most animal tissue, and the demonstration of a complete identity of the two would have important metabolic connotations.

In view of the importance of the field, as well as of the interest of one of us<sup>5</sup> in the problem of carbohydrate utilization, we decided to attempt an extension of the work of von Euler and Myrbäk on the purification of cozymase. At the beginning, however, we found that our yeast produced much less active enzyme preparations than those of von Euler and Myrbäk, as regards both the zymase and cozymase content, and also that the technique of the above authors with lead precipitation, which enabled them to secure an initial purification of from ten- to thirtyfold, was in our case practically useless.

We therefore abandoned our original intention and have instead studied the purification produced in our material by a variety of reagents. In the investigation we have repeated much of the work done by von Euler and Myrbäk, and several differences have been found, which appear difficult to explain solely upon the basis of the lower initial purity of our material. As certain of the experiments show distinct promise, we hope to be able to extend the work upon a material of considerably higher original purity, such as was employed by von Euler and Myrbäk.

#### *Materials and Technique of Assay.*

The yeast which we employed throughout was a bottom yeast supplied by the Eastside Brewery<sup>6</sup> in Los Angeles. It was obtained fresh, transported immediately to Pasadena, and used the same day.

For analyzing extracts for cozymase activity, the analytical procedure adopted was similar to that employed by von Euler and Myrbäk. When zymin (acetone-dried yeast) is washed with water the cozymase is largely removed. The washed material is therefore a source of zymase practically free from cozymase, and

<sup>4</sup> Meyerhof, O., *Z. physiol. Chem.*, 1918, ci, 165; 1918, cii, 1.

<sup>5</sup> Raymond, A. L., *Proc. Nat. Acad. Sc.*, 1925, vii, 622.

<sup>6</sup> I wish to express my gratitude to the many persons connected with the brewery, whose continued courtesy and assistance facilitated this research.  
A. L. R.

on addition of the latter is again capable of producing fermentation. Von Euler and Myrbäck found on adding cozymase extracts to such washed zymin that within reasonable limits of concentration the maximum rate of carbon dioxide production was proportional to the amount of extract added. Our experience in general confirmed this, as well as the fact that it is necessary to keep the phosphate concentration constant throughout. All solutions were adjusted to pH 6.3 to 6.5 before testing, but even in those cases in which all precautions were observed, we found variations of 5 to 10 per cent in certain analyses and our assays are therefore only reliable within these limits.

In order to prepare our zymin the procedure of Albert<sup>7</sup> was used. Although it appears quite simple, we found difficulty in preparing an actively fermenting zymin from our yeast. On substituting technical acetone for c.p. material, a completely inactive preparation resulted, as was the case when the acetone was added with stirring to the yeast instead of the reverse. Even those lots which were prepared by apparently identical procedures showed considerable differences in activity. The experimental work to be reported was, therefore, performed with two very similar lots which were, incidentally, the best we secured.

For assay purposes, the zymin was freed of cozymase by mixing with 8 parts of water and centrifuging. This washing was repeated twice more, and the zymin then produced almost no fermentation in the absence of added cozymase. The zymin was freshly washed as needed. Zymophosphate, prepared from the first zymin washes, was added to remove an induction period which resulted with the purer fractions.

The final concentrations in the fermenting mixture were:

Na <sub>2</sub> HPO <sub>4</sub> .....	0.013 M
KH <sub>2</sub> PO <sub>4</sub> .....	0.020 "
Glucose.....	10.0 per cent
Gentian violet.....	0.125 " "
Zymin (added).....	10.0 " "

The gentian violet was added as an antiseptic and at the above concentration we observed no growths for the periods over which we worked (2 to 12 hours) and there was no appreciable inhibi-

<sup>7</sup> Albert, R., Buchner, E., and Rapp, R., *Ber. chem. Ges.*, 1902, xxxv, 2378.

tion of the enzyme action. The pH of the mixture was 6.4, and the total phosphate concentration was optimum for our yeast.

For determining the carbon dioxide formed an electrometric method<sup>8</sup> devised for the purpose was employed. Readings were made at 15 minute intervals, and the maximum rate was taken as a measure of the cozymase activity of the sample.

Typical fermentation curves are given in Fig. 1. In these the rates of carbon dioxide production are plotted as a function of

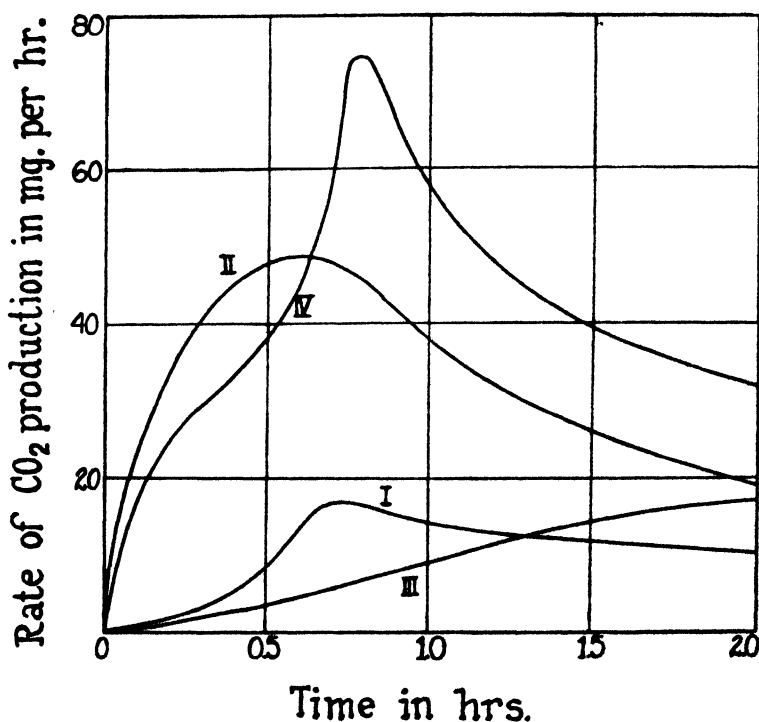


FIG. 1.

the elapsed time. Curves I and II were obtained with different quantities of cozymase, using the glucose-phosphate mixture described above, while Curves III and IV are the corresponding ones for runs employing twice the concentration of phosphate, other factors being unchanged. The longer periods before attainment of the maximum rate in the latter case are characteristic of high phosphate concentrations, as are the larger maximum rates for the same cozymase concentration.

<sup>8</sup> Raymond, A. L., and Winegarden, H. M., *J. Biol. Chem.*, 1927, lxxiv, 189.

For convenience in reporting our results, we have defined a unit as being that quantity of cozymase required to produce a maximum rate of fermentation of 1 mg. of carbon dioxide per hour. We have also used von Euler's activity coefficient, which is defined as the maximum rate of carbon dioxide production in cc. per hour, divided by the solid content of the sample expressed in gm. The symbol Aco was suggested by von Euler to designate this unit and has been employed throughout this paper.

### *Extraction of Cozymase from Yeast.*

Our cozymase solutions were prepared by extracting yeast with hot water, a procedure which has been shown by others to destroy the zymase activity without appreciably impairing the cozymase. Fresh and washed yeasts were both investigated, but as the only difference between the two extracts lay in the fact that those from the washed yeast contained slightly less solids, the latter were not extensively employed.

In order to obtain an efficient extraction, various factors were examined with a view to determining the optimum yield of cozymase, without regard to the purity of the resultant product. Batches of 10 to 20 liters were prepared by adding yeast to water at 90–92°, stirring 2 to 3 minutes, and cooling as rapidly as possible by immersing the metal container in running water.

The effect of concentration of the yeast upon the yield was studied and it was found that the efficiency of the extraction increased largely with decreasing concentration down to 0.1 gm. of fresh yeast per cc. of solution. More dilute extracts were not examined, as solutions of much lower activity required too extensive evaporation before they could be used.

Acidity was also examined with regard to its effect on the yield, glacial acetic acid and sodium carbonate being added to the hot water to vary the acidity. The pH was determined and the solution neutralized to pH 6.4, and tested. The results are included in Table I.

### *Electrodialysis.*

No attempts appear to have been made toward purifying cozymase by means of electrodialysis. As the method offers great possibilities with those substances which migrate under the influ-

ence of an electric field, we decided to investigate the results with our material.

A cell was built consisting of seven 50 cc. compartments, in a row, and separated by parchment paper partitions. There were placed in the compartments in order: I, copper sulfate solution (1 M) and a copper electrode; II, potassium chloride (0.1 M); III, potassium chloride (0.1 M) adjusted to the desired pH; IV, cozymase solution similarly adjusted; V, VI, and VII were duplicates of III, II, and I respectively. The potassium chloride solution in II and VI prevented contamination of that in III and V.

To see if the pH of the solution affected either the magnitude

TABLE I.

Reagent.	HOAc		Control.	Na <sub>2</sub> CO <sub>3</sub>	
Concentration, gm. per liter.....	5	1		1	5
pH of extract.....	3.8	4.6	6.0	7.0	10.0
Activity, units per cc.....	3.6	3.6	4.7	2.9	2.5

TABLE II.

Experiment No.	pH	Activity (units per cc.).		
		Anode.	Center.	Cathode.
IV	2.0	0.2	1.4	0.3
II	4.4	0	2.7	0
I	6.4	0.2	3.2	0.2
III	9.7	0	2.3	0

or direction of migration of the activity, a set of four experiments was performed covering a pH range of 2 to 10. A potential drop of 4 volts per cm. was maintained in the cozymase solution and 1000 to 1800 coulombs passed in 40 to 60 minutes. In the case of Experiment III, the apparatus was kept in the ice box to decrease the destruction due to the alkalinity. The pH did not change appreciably during the run. The activities of the solutions are given in Table II. In no case therefore was an appreciable amount of cozymase transferred to the potassium chloride compartments, although tests showed the parchment paper was permeable to the cozymase over the entire pH range employed.

In view of the fact that the paper was permeable to the cozymase and yet practically no transference occurred in an hour, it seems justifiable to conclude that the cozymase carries no appreciable fraction of the current over the pH range examined. Whether this is due to its being present at low concentration, or to its having a low degree of ionization, or to both, cannot be determined from the above data. However, the possibility of purifying crude solutions by this means seems to be definitely excluded.

#### *Alcohol Precipitation.*

As alcohol has been suggested for purifying cozymase, we performed a number of experiments with this reagent. We found that an alcohol concentration of 40 per cent produced a fine flocculent precipitate and left the majority of the activity in the

TABLE III.

	Yield.	Aco.	
		Dry.	Ash-free.
	<i>per cent</i>		
40 per cent precipitate.....	11	15	29
80 " " ".....	51	36	42
90 " " filtrate.....	61	31	44
Original.....		26	36

solution, but that 80 per cent alcohol, on the other hand, produced a less satisfactory separation and frequently occasioned considerable losses of activity. In no case, however, was there any significant purification.

A typical experiment in which the concentration was first made 40 per cent and then 80 per cent is given in Table III. The yields are expressed as the percentage of the original cozymase present in the different fractions. The failure of the activities to add up to 100 per cent is presumably due either to varying phosphate concentrations in the different fractions, or else to the removal of some inhibiting material. The phenomenon was frequently observed.

#### *Lead Precipitation.*

Due to the great success of von Euler and Myrbäk in using lead precipitation as a means of purification, we attempted to repeat

their experiments with this reagent. The procedure evolved by them consisted in a precipitation at pH 6, which left the active material in the filtrate, followed by precipitation of this active material at pH 10.

We employed both washed and unwashed yeast and prepared an extract exactly as they described. Precipitation at pH 6 gave a mixture very difficult to filter, which was turbid after centrifugation. Practically none of the activity was to be found in the precipitate. Solid determinations indicated only very slight increase in the Aco. Von Euler and Myrbäk, on the other hand, obtained threefold purification and reported no mechanical difficulties.

At pH 10 we found the activity to be again almost entirely in the filtrate. This is contrary to the results of the above authors, who found precipitation to begin at pH 8.5 and be complete at pH 10. We also duplicated our own results with pH 6 filtrates to which additional lead acetate had been added, indicating that the difference in the alkaline precipitation was not due to insufficient lead. To ascertain whether we were dealing solely with a concentration effect, we evaporated a sample of our material to  $\frac{1}{6}$  volume *in vacuo* and repeated the experiment. Again the activity was to be found almost completely in the filtrates, both at pH 6 and 10.

In order to determine the effect of higher pH on the precipitation of the active material, we added lead acetate in different concentrations and tried pH values of 10.5 and 11. The behavior was quite variable and appeared to depend upon the particular batch of material employed. One lot, for example, made 0.005 M with lead acetate, gave a precipitate containing 79 per cent of the active material at pH 10.5 and 72 per cent at pH 11.0, while another, using lead ion concentrations of 0.005, 0.01, and 0.015 M, at pH 10, 10.5, and 11 gave a maximum precipitation of 27 per cent. It is possible that the differences between von Euler's results and our own regarding the precipitation at pH 10 are dependent upon the same factors that must be operating in the cases cited. In any event, the lead is not a satisfactory precipitant until these apparent anomalies are understood.

In those cases in which the lead did precipitate the active material, the purity was considerably increased. The most effective

purification which we secured, however, was but sevenfold increase over the original material. In view of the fact that similar precipitations in the hands of von Euler and Myrbäck produced ten- to thirtyfold increase over their original, the above results do not appear particularly encouraging.

In view of these facts, as well as the one mentioned above that the precipitation is not at all satisfactory at pH 6, we decided to modify von Euler's first procedure as follows. Batches of cozymase solution were prepared by adding 2½ kilos of fresh yeast to 15 liters of water at 90–92°. The solutions were filtered immediately and cooled. The cooled, filtered solutions were placed in a large glass container capable of holding 25 to 30 liters and a hot concentrated lead acetate solution was added to the extent of 7.5 gm. of lead acetate per liter. The pH was then adjusted to 9.0 by the addition of 6 N NaOH with constant stirring, and the mixture was allowed to settle for 1 or 2 minutes. The supernatant portion should be perfectly clear and the mixture should filter rapidly, giving a light yellow, crystal-clear solution. When this was not the case, additional portions of 0.5 gm. per liter of lead acetate were added and the pH readjusted after each addition to 9.0. In no case was it necessary to add more than 8½ gm. per liter of lead acetate to produce perfect precipitation. The phosphate content<sup>9</sup> of the solution dropped from an average of 0.35 mg. per cc. to about 0.007 mg. per cc. which accounts for a large portion of the added lead.

These final solutions, after neutralization and removal of lead, contained from 90 to 100 per cent of the activity of the original extract but solid determinations showed only slight changes in Aco.

Solutions prepared in this way and still containing a small amount of lead, were quite stable. Unlike the original boiled yeast extracts, they exhibited little tendency to develop mold or bacterial growths, and lost less than 50 per cent of their activity on being kept in the ice box for 2 or 3 weeks. As mentioned above, inorganic phosphates were almost completely removed by the lead precipitation, and therefore did not interfere with additional reagents. For these reasons, we adopted this precipitation as

<sup>9</sup> Determined by the method of Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, lxi, 63.



standard and the further work described in this paper was largely done on such lead-treated solutions, from which excess lead was not removed.

### *Mercury Precipitation.*

The results with mercury as a precipitant for cozymase were particularly interesting, as they showed certain peculiarities that do not appear to have been reported previously in biological purifications. On adding  $\text{Hg}(\text{CN})_2$ ,  $\text{HgCl}_2$ , and  $\text{Hg}(\text{NO}_3)_2$  of the same concentration to the lead-treated solution, there were observed, respectively, no, slight, and heavy precipitation. This is in exact correspondence with the degree of ionization of these salts.

More quantitative experiments were performed with  $\text{HgCl}_2$  and  $\text{Hg}(\text{NO}_3)_2$ . To samples of cozymase solution  $\text{HgCl}_2$  solution was

TABLE IV.

HgCl <sub>2</sub> concentration.	Yield.	Aco.	
		Dry.	Ash-free.
<i>M</i>	<i>per cent</i>		
Original.		20	31
0.0025	25	75	139
0.005	42	125	185
0.0075	65	117	192

added to concentrations of 0.0025, 0.005, and 0.0075 M and the pH was adjusted to 7.8 to 8.0. The addition of a few drops more of  $\text{HgCl}_2$  solution produced no precipitation or opalescence in the filtrates, indicating complete precipitation in each case. It was therefore of great interest that not only did the yield of active material carried down by the precipitate increase with increasing concentration of  $\text{HgCl}_2$ , but the Aco likewise progressively increased. The data are given in Table IV.

The above experiment was then repeated with  $\text{Hg}(\text{NO}_3)_2$  at the same concentrations. The clear solutions, after centrifuging off the precipitate, were tested with more of the reagent and further precipitation was found to occur with the 0.0025 and 0.005 M concentrations, but not with the 0.0075. In this case, the yields increased as before, but the purity underwent a constant decrease.

In experiments at pH 6.5 the results were exactly similar to those at the higher pH, but the yield was in each case lower.

The results confirm the experiment of von Euler to the extent that  $\text{HgCl}_2$  to complete precipitation in neutral or acid solution is a poor precipitant. They are, however, quite different in their final interpretation, for they indicate  $\text{HgCl}_2$  to have considerable possibilities in the purification, if used in larger quantities than necessary for complete precipitation.

The experiments are also of importance in connection with biological purifications employing mercury, as they indicate important differences resulting from different salts of this element, and serve as well to invalidate complete precipitation as a reliable guide.

#### *Silver Precipitation.*

Silver, like mercury, was found to be a fairly satisfactory precipitant for cozymase. Our preliminary experiments indicated

TABLE V.

Concentration, <i>M</i> .....	0.0	0.025	0.005	0.0075
Yield, <i>per cent</i> .....		48	61	63
Aco (ash-free).....	30	173	206	203

approximately neutral solutions to be satisfactory and the effect of concentration was therefore investigated at pH 7.2 to 7.3. One experiment is listed in Table V.

Attempts to increase the above yields by altering the acidity met with failure. Occasional batches, however, gave yields of 75 to 85 per cent at the above pH. As the Aco was likewise fairly high, as will be observed above, it would appear that silver might perhaps be profitably employed.

#### *Removal of Metals as Sulfides.*

In the case of the three metals described above, lead, mercury, and silver, it was frequently observed that the loss in total cozymase was very great. This was assumed to be due to the fact that when the metals were removed as sulfides, as a preliminary to the tests of activity, some of the cozymase was adsorbed on

the precipitated sulfides. An experiment was undertaken to examine this possibility.

To pairs of samples of cozymase solution the above three ions were added to a concentration of 0.0075 M, and the pH was adjusted in one of each pair to 10 and in the other to 5. Without removing any precipitate, the solutions were saturated with hydrogen sulfide, filtered, and the hydrogen sulfide removed *in vacuo*. The solutions were then tested and the losses in activity thus determined. Losses of from 10 to 20 per cent were observed in the acid solutions and from 0 to 10 per cent in the alkaline.

In purer samples the above phenomenon was of even greater magnitude. With silver, in particular, we had great difficulty and in a few cases losses of as high as 60 and 80 per cent occurred. As the precipitation of the sulfides is best accomplished in acid solutions and as it is here that the greatest losses occur, this factor must be given close attention. We avoided the difficulty as best we could by precipitating the sulfides from dilute solutions in which case the losses were much lower. The matter is of interest because of its general bearing on the problem of biological purifications by means of the heavy metals.

#### *Cadmium.*

Cadmium hydroxide was precipitated by adding  $\text{CdCl}_2$  to the cozymase solution to the desired molality, adjusting to pH 10.5 to 10.7, centrifuging off the precipitate, neutralizing both portions, and removing the cadmium as a sulfide. Preliminary experiments had shown practically no losses by adsorption on cadmium sulfide. Not only were the yields invariably low, but also almost no purification resulted, and this was true at both lower and higher acidities.

#### *Iron.*

As precipitation of iron hydroxide in the cozymase solutions was found to be difficult, the hydroxide was freshly prepared, washed, and added to the cozymase solution to 0.05 molal. The solution was made alkaline to pH 10.1 to 10.2, and shaken for 10 to 20 minutes. On centrifuging off the precipitate, neutralizing, and testing, 88 per cent of the original activity was found to remain in the filtrate, while the precipitate was inactive.

*Aluminum.*

The use of aluminum hydroxide as an adsorbent for the cozymase activity has been discussed by von Euler. He found that the hydroxide was a quite effective adsorbent at pH 10, and deduced the Aco of the material to be quite high. He did not, however, utilize the method.

On original extracts, untreated with lead, we found aluminum hydroxide to be quite ineffective. Aluminum sulfate was added to aluminum ion concentrations of 0.005, 0.01, and 0.015 molal, and then NaOH to pH 10. The precipitates were centrifuged off and both portions neutralized. Table VI gives the distribution of the cozymase in per cent of the original. However, in repeating the experiment, with the exception that a lead-precipitated

TABLE VI.

Concentration, <i>M</i> .....	0.005	0.01	0.015
Precipitate, <i>per cent</i> .....	3	7	25
Filtrate, <i>per cent</i> .....	90	61	59

TABLE VII.

Concentration, <i>M</i> .....	0.005	0.01	0.015
Precipitate, <i>per cent</i> .....	77	103	75
Filtrate, <i>per cent</i> .....	33	4	0

solution was employed, more promising results were secured, as indicated in Table VII.

The difference in the behavior of the two solutions may perhaps be accounted for by the absence of phosphate in the lead-precipitated material, as aluminum phosphate, which is surely formed in the original material, may interfere.

The problem of recovering the active material from the hydroxide still remained. Two methods were examined and both found to be useful. The first consisted in dissolving the precipitate in a minimal quantity of 6 *N* hydrochloric acid, diluting, and neutralizing. In the second method the precipitate was suspended in water and adjusted to pH 5.9 to 6.1 and kept in the ice box for 12 to 24 hours. As the mixture became more alkaline on standing, it was readjusted once or twice during the period.

Both methods were found to give filtrates containing the major portion of the active material. The second procedure, moreover, resulted in a fairly pure product, the Aco being increased, for example, in one experiment from 30 to 275 (ash-free) by precipitating at pH 10 and reversing the adsorption at pH 6.0. Repeating the adsorption and reversal once more caused an increase to only 335.

#### *Miscellaneous Reagents.*

The remaining reagents which we employed were examined only casually and will, for this reason, be only briefly mentioned.

Barium, in accordance with the statements of Von Euler, was found to produce no precipitation of the active material, even in a solution as alkaline as pH 10.5.

Tannic acid was likewise found to precipitate none of the active material from dilute solutions, and concentrated solutions were not investigated. The filtrate from the tannic acid precipitation was noted to develop only faint precipitates with either phosphotungstic or silicotungstic acids.

Silicotungstic and phosphotungstic acids were both tried, but without particular success. Considerable loss of activity in general resulted with no very great increase in the purity of the cozymase preparation.

We desire to thank Professor A. A. Noyes for grants and facilities which allowed us to pursue these studies, and Professor R. C. Tolman for his constant interest and encouragement.

#### SUMMARY.

Electrodialysis and a number of reagents have been examined with regard to their applicability to purifying cozymase extracts.

Lead salts were found to give variable results. The experiments of von Euler and Myrbäck could not be duplicated on the available cozymase extracts.

Mercuric chloride and nitrate were found to effect different results. The experiments appear to have considerable bearing on biological purifications with this element.

Mercury, silver, and aluminum were found to be most useful.

## THE DETERMINATION OF CARBON DIOXIDE IN FERMENTING MIXTURES.\*

By ALBERT L. RAYMOND† AND HOWARD M. WINEGARDEN.

(From the Gates Chemical Laboratory, California Institute of Technology,  
Pasadena.)

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### INTRODUCTION.

In connection with a research on enzymic behavior it was necessary to develop a simple and expeditious method for the determination of the carbon dioxide formed during fermentations. The most suitable method of carbon dioxide analysis appeared to be the procedure evolved by Cain and Maxwell<sup>1</sup> for the determination of carbon in steel. They absorbed the carbon dioxide, formed by combustion, in known volumes of barium hydroxide solution and followed the precipitation of barium carbonate by measurements of the electrical conductivity of the solution. The idea has been applied by Spoehr and McGee<sup>2,3</sup> to the determination of carbon dioxide in their studies on plant respiration. This paper describes the development of this general method so as to render it applicable to studies on fermentation.

The technique which was finally adopted consisted in liberating the carbon dioxide from the fermentation mixtures by shaking, carrying it by means of a stream of air free from carbon dioxide to the absorption vessels containing barium hydroxide, and measuring the change in resistance of the barium hydroxide solutions.

\* Contribution No. 123 from the Gates Chemical Laboratory, California Institute of Technology, Pasadena.

† National Research Fellow in Chemistry.

<sup>1</sup> Cain, J. R., and Maxwell, L. C., *J. Ind. and Eng. Chem.*, 1919, xi, 852.

<sup>2</sup> Spoehr, H. A., and McGee, J. M., *Ind. and Eng. Chem.*, 1924, xvi, 128.

<sup>3</sup> Spoehr, H. A., and McGee, J. M., *Carnegie Institution of Washington, Pub. No. 325*, 1923.

*Conductivity of Barium Hydroxide Solutions.*

Before undertaking the carbon dioxide determinations, it was necessary to determine the conductivities of pure barium hydroxide solutions. The conductivities found in the literature are almost exclusively at 25° and are not in very good agreement. As we wished to employ both 30° and 37°, we determined the conductivity at these temperatures over the concentration range of 0.065 to 0.12 N, and in addition repeated the observations at 25° for comparison with previously published data.

In making these determinations the usual precautions were observed. Conductivity water of specific conductance of from 0.8 to  $1.2 \times 10^{-6}$  mhos. was prepared by redistilling the laboratory supply of distilled water, first from acid permanganate and then from barium hydroxide, all in a current of air free from carbon dioxide. The potassium chloride employed as a conductivity standard was Baker's c.p. twice recrystallized. The barium hydroxide was Merck's c.p. similarly treated. Sodium carbonate was used as an acidimetric standard and was prepared by gentle ignition of pure sodium bicarbonate, which in turn was secured by saturating with carbon dioxide a cooled solution of Baker's c.p. sodium carbonate. Various samples of the final carbonate prepared as above gave reproducible analyses. Baker's c.p. hydrochloric acid was diluted and standardized against the sodium carbonate, using methyl orange as indicator. In all titrations the neutral solution was boiled to expel carbon dioxide, cooled, and the end-point redetermined. The final value chosen for the normality of the hydrochloric acid was the average of five determinations in which the greatest difference was 0.15 per cent. The barium hydroxide solutions were standardized against this hydrochloric acid using the average of three or four determinations, which in general showed differences of not more than 0.25 per cent.

The temperature of the thermostat was read to  $\pm 0.01^\circ$  by means of a standardized thermometer. Barium hydroxide solutions of various concentrations were immersed in the thermostat, and after allowing them to come to temperature their resistances were determined with a dip electrode. The thermostat temperature was then changed and the determinations repeated.

Measurements were made on each sample at 25°, 30°, and 37°. At each temperature the cell constant of the electrodes was determined by immersion in an accurately prepared potassium chloride solution both before and after measuring the conductivities. In no case did the cell constant undergo appreciable change during the measurements.

After several preliminary experiments the data given in Table I were secured in three runs, as indicated in the first column. Each sample was used at the three temperatures and the normali-

TABLE I.

Run No.	Normality at 20°.	CO <sub>2</sub> per 100 cc.	Specific resistance.		
			25°	30°	37°
		mg.			
I	0.1131	248.9	43.43	40.13	36.16
	0.1000	220.0	48.78	45.04	40.60
	0.0907	199.6	53.21	49.12	44.30
	0.0817	179.9	58.63	54.12	48.85
	0.0723	159.1	65.40	60.33	54.48
	0.0633	139.3	73.68	68.13	61.48
II	0.1039	228.6	47.05	43.45	39.25
	0.0954	209.8	51.00	47.07	42.49
	0.0862	189.6	55.91	51.64	46.61
	0.0771	169.7	61.90	57.15	51.57
	0.0680	149.5	69.49	64.15	57.88
	0.1172	257.9	42.12	38.94	35.15
III	0.1092	240.2	44.89	41.50	37.41
	0.0910	200.3	53.15	49.11	44.32
	0.0711	156.4	66.70	61.52	55.58

ties given in the second column are those which the solutions would have at 20°. For convenience in our later work the concentrations were recalculated and expressed in mg. of carbon dioxide per 100 cc. of solution, by which is meant the mg. of carbon dioxide stoichiometrically equivalent to the barium hydroxide in 100 cc. of solution. Thus 0.1 N solution is expressed as 220 mg. of CO<sub>2</sub> per 100 cc. These values appear in the third column. The last three columns in the table give the specific resistances as calculated from the observed conductivities and cell constants.

For comparison with the data of Cain and Maxwell<sup>1</sup> and Spoehr



and McGee<sup>2,3</sup> they are all plotted in Fig. 1. A single point falling in this region determined by A. A. Noyes<sup>4</sup> is included.

*Apparatus and Method.*

The electrical equipment for the determination of conductivities differed but slightly from that employed by Cain and Maxwell.<sup>1</sup>

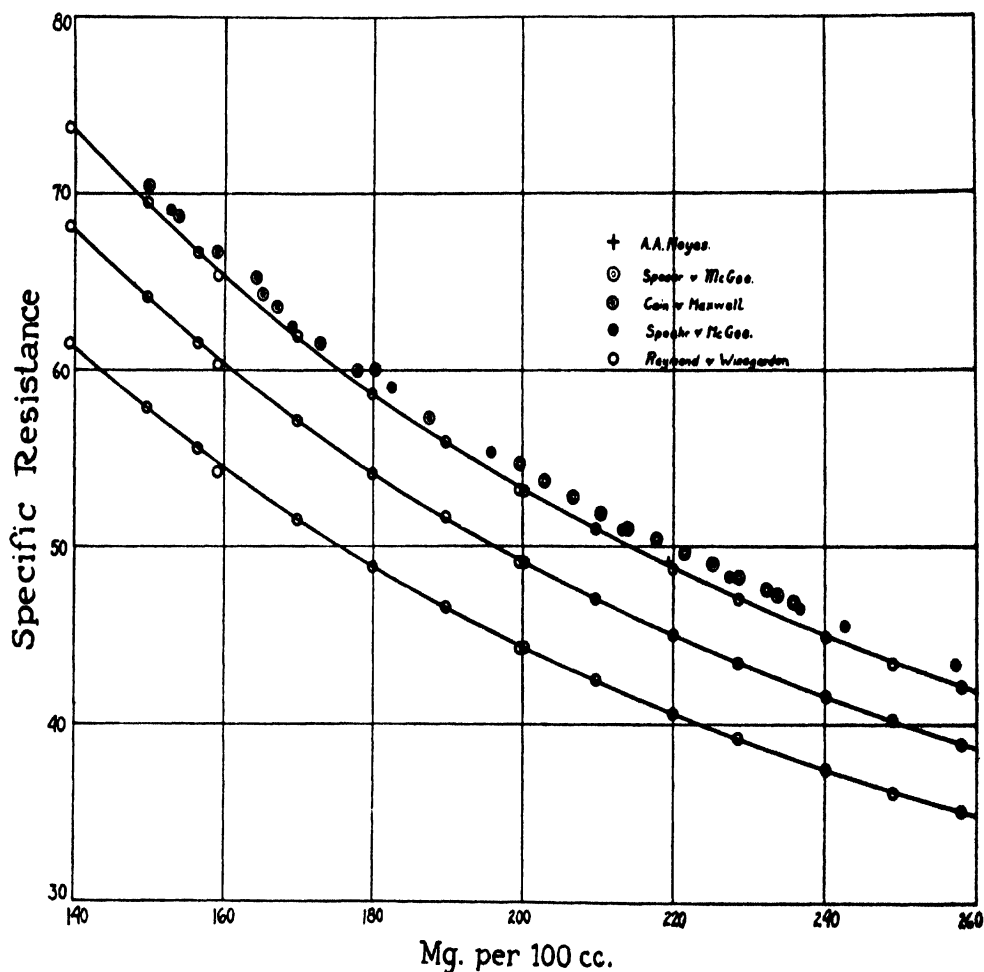


FIG. 1.

A Weibel<sup>5</sup> type A.C. galvanometer operating on the 110 volt 50 cycle light circuit supplied 6 volts A.C. for the bridge. The

<sup>4</sup> Noyes, A. A., *Carnegie Institution of Washington, Pub. No. 63, 1907, 254.*

<sup>5</sup> Weibel, E. E., *Bureau of Standards, Scientific and Technical Papers, No. 297, 1917.*

galvanometer was of low resistance, and we therefore substituted a low resistance Kohlrausch bridge and a four dial Curtis box for their self-contained bridge. An advantage of this change will be discussed later. The instruments were manufactured by Leeds and Northrup and gave complete satisfaction. Resistances up to 1000 ohms could be measured to within 0.05 per cent, which was greater accuracy than we required. Carbon dioxide determinations were made with an accuracy of  $\pm 0.0006$  mg. per cc. of absorbing solution or  $\pm 0.15$  mg. for a 250 cc. cell.

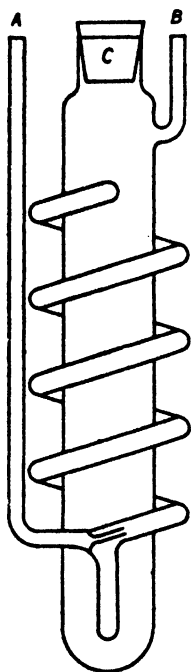
For convenience as well as for reproducibility and accuracy in the carbon dioxide determinations, it was decided at the outset to immerse both the fermentation vessels and barium hydroxide solutions in a single large thermostat. One equipped with the usual stirrer and regulator and capable of maintaining a desired temperature to within  $\pm 0.02^\circ$  was employed throughout.

For transferring the carbon dioxide to the absorption vessels a stream of air free from carbon dioxide was used. The air was purified by passing through a calcium chloride tower and then through three soda-lime towers in series. By refilling the most used one of these every 3 to 4 weeks and moving the towers in a counter-current fashion, no trouble was experienced from carbon dioxide in the gas stream. A pressure-regulating device consisting of an 8 inch U-tube about one-fourth filled with mercury was connected to the air line by means of a large T-tube. If the line pressure exceeded the desired value, the gas stream escaped through the mercury. This was found to give as close regulation as was necessary. To maintain constant rates of gas flow a piece of glass capillary tube was placed in the line following the pressure regulator. By suitably choosing the size of the capillary and the amount of mercury in the U-tube, the gas flow was easily adjusted to any desired value. For our work we found 200 cc. of air per minute to be satisfactory. An exactly constant rate of gas flow is, however, probably unnecessary, as will be indicated later.

After trying several types of gas absorbers we decided that the design of Weaver and Edwards<sup>6</sup> was best suited to our purpose. It is a compact and very efficient absorber, even at high rates of gas flow. Their original design was simplified and the lower

<sup>6</sup> Weaver, E. R., and Edwards, J. D., *J. Ind. and Eng. Chem.*, 1915, vii, 534.

stop-cock omitted, as illustrated in Fig. 2. The absorber is for 250 cc. of solution but may be altered to permit the use of any quantity. The gas stream enters through the tube *A*, carries the absorbing liquid up the spiral, and finally escapes through the outlet *B*. The spirals were built of tubing which was 8 mm. inside diameter and although considerable precipitation of barium carbonate occurred in them, the absorbers could be used for several runs before cleaning and refilling. A difficulty which has been



Scale in cms.

FIG. 2.

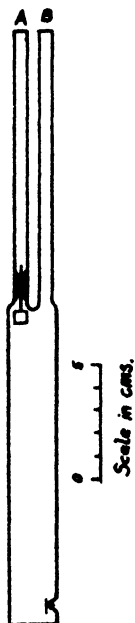


FIG. 3.

experienced, however, is that the injector may become clogged during a run. To avoid this the constriction can be omitted as the bubbles are still small enough to produce efficient absorption at low rates of gas flow. A soda-lime tube was attached to the small upper outlet of the absorber to prevent carbon dioxide entering from the air.

A further advantage of this type of absorber is that it is admirably suited to the use of pipette conductivity cells, which were inserted through the rubber stopper *C* and left in place. The

cell which we employed is illustrated in Fig. 3. The upper electrode is sealed into the tube *A*, which also serves to insulate the lead wire. The connecting wire to the lower electrode is stretched along the side of the cell and held in place by the 2-hole stopper into which the two tubes are inserted. The open tube *B* serves to admit air free from carbon dioxide, in order to blow out the contents of the electrode as well as to mix the solution in the absorber. The cell is immersed so that the upper electrode is below the liquid level and the pipette need therefore only be blown out, as it refills of itself. It was found by blowing out the contents three or four times that the solution was uniform and further mixing produced no changes in resistance. A 3-way stop-cock was connected to a source of air free from carbon dioxide in such a

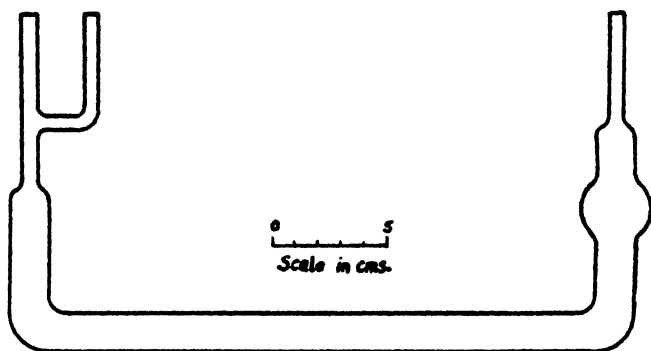


FIG. 4.

way that the electrode might be alternately blown out and allowed to refill by merely turning the stop-cock.

In order to liberate the carbon dioxide from the fermenting mixtures it was desirable to expose as large a liquid surface as possible to the gas stream. For this reason the fermentation tube illustrated in Fig. 4 was employed, the horizontal part of the tube being about half filled by 20 to 25 cc. of solution. Solutions could be added or withdrawn through the straight tube with a pipette without discontinuing the current of air free from carbon dioxide which entered through the branch tube. The straight tube was capped, except during such removals or additions. In order to accelerate the evolution of carbon dioxide, the tube was fastened with small clamps to a rocking table immersed in the

thermostat. The table was oscillated by means of a small electric motor fitted with a worm drive and so arranged that both the frequency and the amplitude of the oscillation could be changed. After some preliminary experiments an amplitude of  $\pm 6^\circ$  from the horizontal and a frequency of about 120 per minute were adopted.

In our final apparatus we mounted six identical sets side by side in the thermostat, the shaking table being built to accommodate six tubes. The air lines for transferring the carbon dioxide and for blowing out the pipette electrodes were manifolded. Gas-washing towers filled with water were placed in the air lines and immersed in the thermostat to saturate the incoming air and prevent evaporation.

For convenience an automatic pipette delivering exactly 250 cc. was used to fill the absorption vessels. A further time-saving device was made possible by our electrical hook up. The cell constants of our different electrodes were in general not the same. However, by setting the four dial resistance box to correspond to the constant of the cell employed (the setting takes but a few seconds) it was possible to compensate for these differences. The strength of the barium hydroxide solutions was then calculated from the data of Table I and plotted as a function of the bridge reading. The graph was found to be very useful, as the calculations were otherwise quite tedious.

#### *Reliability of the Analytical Method.*

The observation of Spoehr<sup>4</sup> that the constant of an immersed cell is subject to change due to precipitation of barium carbonate led us to examine this question. We found that removing the cell from solution and allowing carbon dioxide from the air to form barium carbonate, as suggested by Spoehr, did produce a change in cell constant. However, as long as the electrodes were kept completely immersed no appreciable change in cell constant was observed during a run. This is presumably due to the fact that the barium carbonate is largely precipitated in the spiral, before coming into contact with the electrodes, changes in cell constant being due to the precipitation of barium carbonate upon the platinized surface and not to adsorption of previously precipitated barium carbonate.

It was observed, however, that the resistance of the barium hydroxide solutions increased slightly when kept 24 to 48 hours in our cells. This occurred, nevertheless, even in the absence of carbonate, and was not of sufficient magnitude to introduce appreciable error. The cause of this phenomenon was not investigated.

An additional difficulty which we had anticipated was that the precipitation would be so slow as to require a correction factor. To examine this possibility, resistance determinations were made  $\frac{1}{2}$ , 1, and 5 minutes after introducing carbon dioxide and again after 10 to 24 hours. Changes after 1 minute were found to be negligible as long as the solutions always contained a relatively large excess of barium hydroxide, as was the case in the range of concentrations which we employed.

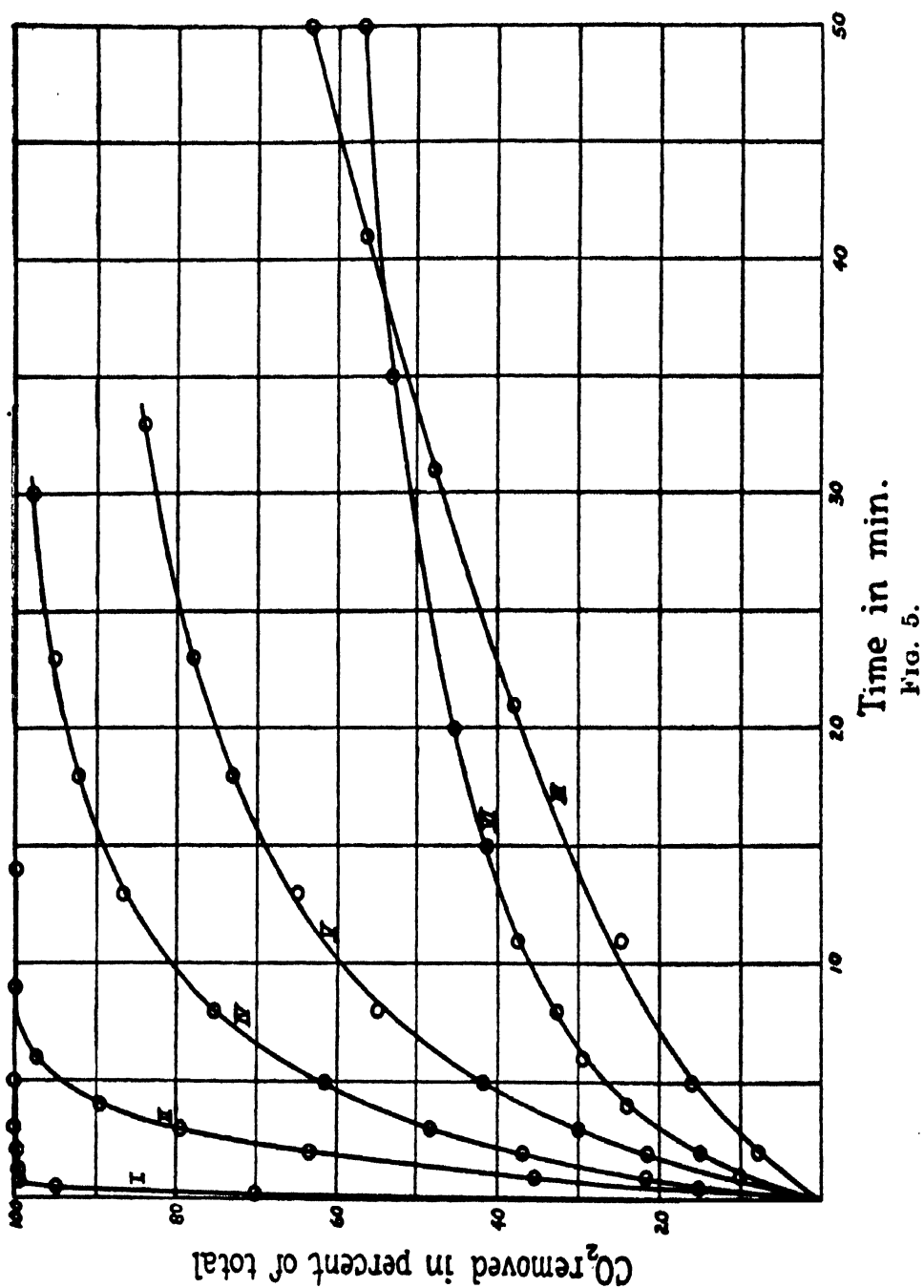
In order to show that the method actually gave correct results on carbon dioxide, we prepared samples of gas by adding measured volumes of standard sodium carbonate solution to an excess of dilute sulfuric acid. A stream of air free from carbon dioxide was bubbled through the mixture and then through the absorber. After 15 to 20 minutes the amount of carbon dioxide as determined by the change in resistance was compared with that from the amount of carbonate used. The differences were always less than 1 per cent, and were systematically in the direction which would indicate that not all the carbon dioxide had been evolved from the acid solution.

#### *Rate of Evolution of Carbon Dioxide from Solution.*

Using the apparatus described above we investigated the rate of removal of carbon dioxide from solutions. Fig. 5 illustrates the results obtained. The graphs represent the carbon dioxide removed in different periods of time, the removal being expressed in per cent of the total amount originally present.

In the first experiment 20 cc. of mercury were placed in the fermentation tube and carbon dioxide introduced in the space above. The shaker was started and the air stream turned on. In  $\frac{1}{2}$  minute 95 per cent of the carbon dioxide was precipitated as carbonate, as shown by Curve I. The total amount present was taken as being the value after 10 minutes, as this was identical with the results at 3 and 5 minutes.

The experiment was then repeated with the exception that 20 cc.



of water saturated with carbon dioxide were substituted for the mercury. In this way Curve II was obtained. For comparison this second run was repeated, except that the shaker was not operated. The readings were continued and the value at 24 hours was taken as representing the total amount present. The data for the first 50 minutes are given in Curve III.

From Curves I and II it may be seen that removal of the gas from the space above the liquid is, for all practical purposes complete in 1 minute, but that the rate at which carbon dioxide escapes from the liquid into the gas space is in comparison very slow. From the data for Curve III the rate of escape from an unagitated liquid was found to be 4 per cent per minute at the start, dropping rapidly to 2 per cent per minute. From the agitated liquid, however, the rate of escape was about 60 per cent of the residual carbon dioxide per minute. This illustrates the extreme importance of the shaking. Increasing the shaking, placing beads in the tube, and using a corrugated tube that broke up the liquid surface more thoroughly, each produced slightly better results, but not enough to compensate for certain mechanical disadvantages. With different mechanical arrangement, which would allow very violent shaking, it should be possible still further to increase the rate of removal.

The rate of gas flow was found, as mentioned above, to be of but slight consequence. By shaking the mixture with the air stream off, stopping the shaking, and turning on the air, a curve almost identical with Curve III was obtained. The average rate in this case was about 55 per cent of the residual carbon dioxide removed per minute.

If we assume 50 per cent per minute to be obtainable it is possible to calculate the limitations of the method. If carbon dioxide is being produced at a constant rate, then the error in observation the 1st minute after the beginning of such production will amount to 50 per cent of the production. The 2nd minute the error will be only 25 per cent, and the 3rd minute only 12 per cent, etc. Similarly, if some acceleration (either positive or negative) in the rate of production suddenly occurs, then after 5 minutes this will be reported to within about 3 per cent of the increment. For small uniform accelerations the error is not appreciable. It may therefore be stated that the method may be used with negligible



errors for uniformly accelerated changes, while for instantaneous changes the error is negligible after 5 minutes. Observations may, therefore, not be made less than 5 minutes after some sudden change in rate. As an example, there may be included the results (Fig. 6) on a fermentation employing live yeast. At the point *P* saturated mercuric chloride solution was introduced. Although carbon dioxide production probably ceased almost immediately, the observations do not report this fact for several minutes, as may be seen from the curve.

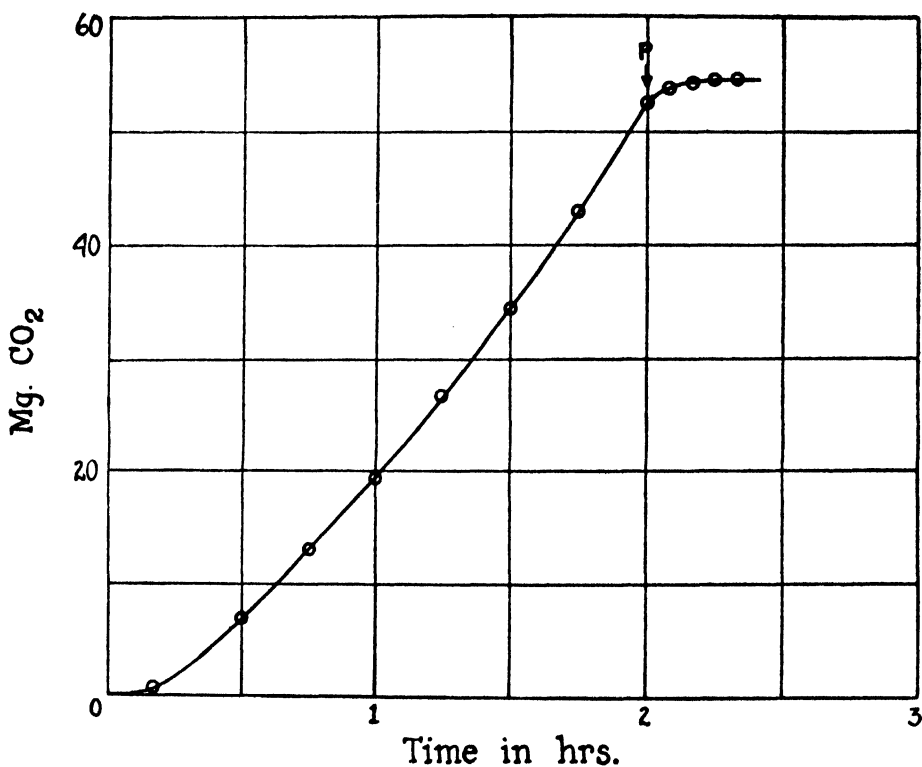


FIG. 6.

A further factor which must be considered in this connection is the pH of the solutions. In the experiments just reported, since the solutions were unbuffered, the pH was between 4 and 7. In this region the carbon dioxide is largely present as carbon dioxide (or  $\text{H}_2\text{CO}_3$ ). If, however, the solutions be made more alkaline, the proportion of carbon dioxide as  $\text{CO}_3^{--}$  and  $\text{HCO}_3^-$  increases, as shown in Table II. Since 50 per cent of the total

carbon dioxide is removed per minute in the acid solutions, it might be expected that 50 per cent of the free carbon dioxide ( $\text{CO}_2$  not present as  $\text{HCO}_3^-$  or  $\text{CO}_3^{--}$ ) would in general be removed per minute. This was investigated experimentally. Buffered solutions were substituted for the water and the shaker was operated throughout. To obtain the total amount originally present, the solutions were acidified after 30 to 50 minutes, and shaken till equilibrium was reached. Curves IV, V, and VI in Fig. 5 represent the results at pH 7, 8, and 9, respectively. It may be

TABLE II.

Per cent of total present as:	pH					
	4	5	6	7	8	9
$\text{HCO}_3^-$ .....	0.3	2.9	23.1	75.0	96.8	93.9
$\text{CO}_3^{--}$ .....			0.001	0.05	0.6	5.6
$\text{CO}_2 + \text{H}_2\text{CO}_3$ .....	99.7	97.1	76.9	25.0	2.6	0.5

TABLE III.

pH	Calculated rate per min.	Observed rate.		
		10 min.	20 min.	30 min.
	<i>per cent</i>			
4	57			
5	55	57		
6	43	42	35	
7	14	12	11	10
8	1.5	6	4	3
9	0.3	2.2	1.2	0.9

seen that the rate of evolution is much less in these more alkaline solutions.

The rates which would be predicted from the fraction present as  $\text{CO}_2 + \text{H}_2\text{CO}_3$  at the different hydrogen ion concentrations were compared with rates actually observed at 10, 20, and 30 minutes. In the calculations it was assumed that 55 per cent per minute is evolved at pH 5 (Table III).

We may therefore conclude that without employing correction factors the method is not easily applicable in its present form to investigations in alkaline media.

*Applications.*

A particular advantage of the method is that it is ideally suited to investigations on aerobic and anaerobic fermentations, as a change from compressed air to compressed nitrogen is all that is required. A disadvantage involved in the application of the procedure to biological studies, however, lies in the difficulty in securing sterility. The tubes may be easily sterilized but the air streams and connections are apt to present difficulties. Our own work has been confined to enzyme systems and we have therefore used an inhibiting agent in the mixture. Toluene, which is most often used for this purpose, is too volatile and we have employed instead a 1:5000 concentration of gentian violet. At this concentration there is no appreciable inhibition of zymase, but no live yeasts have been observed even after 3 or 4 days.

We wish to thank Professor A. A. Noyes for grants which made possible this investigation.

## SUMMARY.

The specific resistances of barium hydroxide solutions from 0.065 to 0.12 N have been determined at 25°, 30°, and 37°.

The rate of the evolution of carbon dioxide from aqueous solutions under various conditions has been examined.

A method has been developed for the rapid and accurate determination of the carbon dioxide formed in fermenting mixtures. The method is applicable to either aerobic or anaerobic investigations. The advantages and limitations of the method are pointed out.

# **OBSERVATIONS ON THE KETONE BODY EXCRETION, THE DEXTROSE TO NITROGEN RATIOS, AND THE GLYCOGEN CONTENT OF LIVER AND MUSCLES OF FASTED, DEPANCREATIZED DOGS.**

By I. L. CHAIKOFF.

*(From the Department of Physiology, University of Toronto, Toronto,  
Canada.)*

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Little attention has hitherto been paid to the excretion of the ketone bodies in pancreatic diabetes in dogs, and it has been stated that ketosis is not so prominent a symptom in this form of diabetes as in the clinical forms. These older observations had to be made on animals immediately following the operation of pancreatectomy, during which time they were being fed with meat, and it seemed of importance to repeat them after removal of insulin from dogs which had been injected with this hormone for a sufficient time following pancreatectomy so that the abdominal wounds had entirely healed and the animal had returned to its preoperative weight and to a satisfactory general bodily condition. In view of the observations made in this laboratory, that the percentage of both sugar and ketone bodies in the blood of depancreatized dogs some days after discontinuance of insulin and food is decidedly higher in fattened, as compared with thin animals, the present observations were planned so that similar comparison could be made with regard to the total excretion of these substances.

In carrying out these observations opportunity was also afforded to accumulate further data regarding the D:N ratio of depancreatized dogs, it having been shown by observations made in this laboratory (Chaikoff, Macleod, *et al.*, 1925, and Macleod and Markowitz, 1926), that this does not usually become stabilized at about 2.8:1, but rather that its mean average for several days, as well as its rate of decline, from day to day, following removal of insulin, depends upon the general nutritive condition of the animal

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at the time the insulin injections are discontinued. These differences could not be accounted for by differences in glycogen stores remaining in the body (liver), but seemed to depend on the amount of body fat. Since the current hypothesis that sugar is derived solely from protein in diabetic animals having no glycogen in the liver, rests mainly on the supposed constancy of the D:N ratio, and since the hypothesis is usually considered to be established by the results obtained mainly by Lusk and his pupils on fasting, or meat-fed, dogs frequently injected with phlorhizin, the importance of additional evidence that the ratio is not necessarily constant in the truly diabetic animal, is self-evident.

### *Methods.*

Pancreatectomy was performed with care to see that all the gland was removed and between experiments the animal was injected twice daily with 16 units of insulin and was fed after each injection with meat, raw pancreas, and cane sugar. When it was desired to fatten the animal, the quantities of each of these foods were 200, 50, and 50 gm., respectively. Otherwise half these quantities were given and the insulin was reduced to 8 units. Under such treatment two dogs, upon which pancreatectomy was performed about  $3\frac{1}{2}$  years ago, are still alive in perfect nutritional condition.

To induce the diabetic state, food and insulin were withheld, and after 24 hours the bladder was emptied and washed by catheter. This urine was discarded and that excreted subsequently was collected from the cage and by catheter at intervals of 12 hours each; this was continued until the 5th day when food and insulin were again given. Sugar was determined by the Shaffer-Hartmann method (1920-21), nitrogen by Kjeldahl, and the ketone bodies by the Van Slyke method (1917); glycogen was determined by Pflüger's method, the muscles being treated with hot KOH (sp. gr. 1.44) and the hydrolyzed glycogen determined as glucose by the Shaffer-Hartmann method.

It may be remarked that the observations on the fattened animals are made difficult because of the persistent vomiting of the animals.

## DISCUSSION.

We will consider first of all the behavior of the D:N ratios.

The average results calculated per kilo of body weight and for 12 hour periods, Tables I and II, may be considered together and they show that decidedly more glucose was excreted by each animal when the body weight was high than when it was low. The nitrogen behavior was not the same in the two animals, being raised by increase in body weight in one of them (Dog C) and lowered in the other. Nevertheless the D:N ratios became higher in both cases with increase in body weight.

In Table III are given the results on two dogs: Dog T which was in fat condition and Dog D in thin condition. In Dog T, it will again be observed that the D:N ratios were high as compared with ratios of poorly nourished dogs.

These results confirm those of Macleod and Markowitz in showing that there is no constancy in the D:N ratio of the same depancreatized animal, but that it varies with the state of bodily nutrition. If we consider the ratios for 24 hour periods commencing in each case with the third one following removal of food and insulin, values of about 2.8 were obtained in Dog F when it weighed 4.8 kilos but they varied between 4.4 and 2.3 when the weight was 5.9 kilos. In Dog C the ratios approached 2.8 after the 3rd day, only when the animal was of reduced weight. If we assume with Minkowski, that 2.8 represents the extent to which sugar is derived from protein in depancreatized animals, then some sugar must have been derived from another source in both animals following liberal feeding. The possible sources are glycogen and fat. With regard to glycogen, that of the liver is doubtless available, but that muscle glycogen might be a source of some of the sugar is improbable, since all the available evidence goes to show that the pathway of the breakdown of muscle glycogen is by way of lactic acid exclusively. Thus Bollman, Mann, and Magath (1925) found that epinephrine did not cause the blood sugar to become increased when it was injected into dogs deprived of the liver, an observation which has been amply confirmed in this laboratory by Soskin (in press) for both epinephrine and anesthetics. Collens, Shelling, and Byron (1926) have also shown that epinephrine causes no change in blood sugar when hypogly-

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TABLE I.  
*Dog F.*

12 hr. periods starting 24 hrs. after withdrawal. Period No.	Ketone bodies calculated as acetone.				Glucose.	Nitrogen.	D:N ratio.		Remarks.
	Acetone and acetoacetic acid.	$\beta$ -Hydroxy- butyric acid.	Total.	gm.			12 hr.	24 hr.	
1	gm.	gm.	gm.	gm.					F <sub>1</sub> , weight 5.9 kilos. Food and insulin Oct. 22.
2	0.031	0.041	0.072	13.80	2.20	6.60			
3	0.031	0.064	0.110	11.10	2.36	4.70			
4	0.048	0.165	0.250	15.60	3.34	4.17	4.43		
5	0.110	0.310	0.420	12.00	3.55	3.38			
6	0.097	0.370	0.470	7.85	3.31	2.37	2.87		
7	0.137	0.510	0.640	7.50	3.26	2.30			
8	0.150	0.550	0.700	7.00	3.12	2.24	2.27		
9	0.140	0.510	0.650	5.20	2.90	1.79			
Total 96 hrs.	0.799	2.520	3.312	80.05	24.04	3.3			
Ratio	1	=	3.15	1.70	0.51				
Average per 12 hrs. and kilo.....			0.07						
1	0.0482	0.172	0.220	5.60	1.07	5.25		F <sub>2</sub> , weight 4.77 kilos. Food and insulin Nov. 14.	
2	0.092	0.445	0.540	4.24	1.57	2.70	2.78		
3	0.120	0.597	0.720	4.10	1.44	2.85			
4	0.206	0.910	1.120	5.62	1.86	3.02	3.09		
5	0.180	0.990	1.180	5.40	1.70	3.17			
6	0.222	0.800	1.020	6.32	1.86	3.40	2.80		
7	0.149	0.455	0.600	3.40	1.54	2.20			
8	0.0316	0.1503	0.080	1.39	1.44	0.096			
9									
Total 96 hrs.	1.05	4.369	5.48	26.07	12.48	2.88			
Ratio	1	=	4.16	0.95	0.325				
Average per 12 hrs. and kilo.....			0.14						

TABLE II.  
Dog C.

12 hr. periods starting 24 hrs. after withdrawal. Period No.	Ketone bodies calculated as acetone.			Glucose.	Nitrogen.	D:N ratio.		Remarks.
	Acetone and acetoacetic acid.	$\beta$ -Hydroxy- butyric acid.	Total.			12 hr.	24 hr.	
	gm.	gm.	gm.	gm.	gm.			
1				14.3	1.75			C <sub>1</sub> , weight 8.63 kilos. Food and insulin Sept. 20.  C <sub>2</sub> , weight 6.36 kilos. Food and insulin Aug. 21.
2				20.3	1.96		9.33	
3	0.0220	0.0364	0.0587	12.4	1.64			
4	0.0343	0.0899	0.124	11.2	2.19	5.10	6.16	
5	0.145	0.384	0.529	12.1	2.67	4.53		
6	0.434	0.988	1.42	17.0	3.21	5.30	4.95	
7	0.830	1.310	2.14	21.2	3.35	6.34		
8	0.781	1.17	1.95	15.0	2.68	5.60	5.97	
9	0.500	0.705	1.20	8.05	1.86	4.45		
Total 84 hrs.	2.746	4.683	7.42	96.95	17.59	5.5		
Ratio Average per 12 hrs. and kilo.....	1 =	1.7	0.123	1.60	0.29			
1				8.60	1.50		6.58	C <sub>2</sub> , weight 6.36 kilos. Food and insulin Aug. 21.
2				11.20	1.51			
3	0.0104	0.0185	0.0289	7.85	1.66	3.29	4.22	
4	0.0265	0.0560	0.0825	6.40	1.95	2.88		
5	0.154	0.422	0.576	7.15	2.48	2.63	2.94	
6	0.217	0.870	1.09	7.80	2.97	3.01		
7	0.400	1.44	1.84	9.10	3.02	2.88	2.94	
8	0.243	0.885	1.13	8.50	2.95	3.18		
9	0.410	1.16	1.47	10.20	3.20			
Total	1.450	4.751	6.217	57.00	18.23	3.12		
Ratio Average per 12 hrs. and kilo.....	1 =	3.2	0.138	1.28	0.41			



TABLE III.  
*Dogs T and D.*

12 hr. periods starting 24 hrs. after withdrawal. Period No.	Ketone bodies calculated as acetone.				Glucose.  gm.	Nitrogen.  gm.	D:N ratio.		Remarks.
	Acetone + acetoacetic acid.	β-Hydroxy- butyric acid.	Lost—vomited.	Total.			12 hr.	24 hr.	
1	0.125	0.170	0.295	26.5	3.85	6.8		T, fat dog, weight 11.36 kilos. Food and insulin withdrawn Sept. 14. Dog vomited. Urine filtered.	
2	0.158	0.207	0.365	17.2	4.10	4.22			
3	0.316	0.465	0.781	17.6	5.58	3.23			
4	0.196	0.265	0.461	9.90	3.02	3.27			
5	Animal found dead.								
6									
Total	0.795	1.107	1.902	71.2	16.55	4.3			
Ratio	1	= 1.38							
Average per 12 hrs. and kilo.....			0.0418	1.56	0.364				
1	0.033	0.041	0.074	14.0	2.45	5.3	5.50	D, thin dog, weight 5.08 kilos. Food and insulin withdrawn July 16.	
2	0.055	0.067	0.122	14.80	2.79	3.08			
3	0.061	0.124	0.185	7.63	2.47	3.03	3.05		
4	0.103	0.223	0.326	9.20	3.04	1.93			
5	0.119	0.275	0.394	6.80	3.52	2.08	2.00		
6	0.147	0.313	0.460	6.80	3.27	2.28			
7	0.120	0.210	0.312	5.10	2.28	2.48	2.38		
8	0.122	0.209	0.331	8.90	3.58				
Total	0.742	1.462	2.204	73.23	23.40	3.13			
Ratio	1	= 2.0							
Average per 12 hrs. and kilo.....			0.0485	1.61	0.51				

cemia sets in following ligation of the hepatic arteries. In post-mortem glycogenolysis, lactic acid appears in place of glycogen in muscle, whereas sugar appears in the liver (Macleod and Simpson, in preparation).

The glycogen of the muscles cannot, therefore, be directly reconverted into blood sugar. It can only be so converted when lactic acid is carried by the blood to the liver.

But even supposing it were thus converted, the evidence shows that the amount of glycogen which disappears is insufficient to account for all the sugar which is excreted on withdrawal of insulin from previously well fed animals, over and above that derivable from protein.

This evidence has been obtained by careful measurement of the glycogen content of the liver and muscles following withdrawal of insulin.

Dogs after pancreatectomy were fed twice daily until the wound had completely healed, on the usual diet of 200 gm. of meat, 50 gm. of pancreas, and 50 gm. of cane sugar, after the injection of 16 units of insulin. Food and insulin were then withdrawn, and after 24 hours the animal was placed in the respiratory cabinet. Animals were guillotined after 3 or 5 days fasting, and the sartorius and gracilis muscles of both sides were quickly dissected out (by cutting across the tendons) and placed in tared flasks containing hot KOH (60 per cent) solution. A portion of liver was also removed and similarly treated. The results are shown in Table IV. In three of the four animals which were killed in 3 days after insulin withdrawal the R.Q's on the 2nd day were 0.70, 0.66, and 0.69 respectively, indicating according to the usual interpretation that there was no direct utilization of carbohydrate. The liver in all four animals contained glycogen in appreciable quantities; *viz.*, 0.23, 0.29, 0.34, and 0.28 per cent, average 0.29 per cent. Unfortunately, the weight of the liver was not determined in these experiments, but assuming it to have been 5 per cent of the body weight, which exceeds the possible maximum, the total amounts of glycogen corresponding to the last three of the above percentages would be, respectively  $\frac{6.7}{20} \times 0.29 = 0.98$

gm.,  $\frac{7.4}{20} \times 0.34 = 1.26$  gm., and  $\frac{9.1}{20} \times 0.28 = 1.27$  gm. In three

TABLE IV.  
*Respiratory Quotient and Liver and Muscle Glycogen of Depancreatized Dogs.*

Dog No.	Weight. kg.	Date depancreatized.	Last day fed, 9 a. m.	Day killed, 9 a. m.	Duration of fasting. days	Date.	R. Q.	O <sub>2</sub> cc.	Glycogen.								Observation of duodenum.	Nutritional condition of dog before death.
									Liver. per cent	Muscle.				Average. per cent				
										Right sartorius. per cent	Right gracilis. per cent	Left sartorius. per cent	Left gracilis. per cent					
1		Nov. 18	Dec. 14	Dec. 17	3		Not done.		0.23	0.41	0.47	0.32	0.28	0.37	No pancreas tissue found.			
2	6.7	Dec. 11	" 27	" 30	3	Dec. 29	0.703	614	0.32 0.26	0.36	0.59			0.47*	"			
5	7.4	" 20	Jan. 10	Jan. 13	3	Jan. 12	0.662	648	0.33 0.35	0.39	0.40	0.42	0.48	0.42	Pancreas tissue with- in duodenal wall, but no islet tissue.	Medium.		
8	9.1	Jan. 14	" 25	" 28	3	" 27	0.687	631	0.27 0.29	0.44	0.50	0.41	0.42	0.44	No pancreas tissue found.	Medium.		
3	5.4	Dec. 11	Dec. 26	Dec. 31	5	Dec. 30	0.682	871	0.075 0.079	0.24	0.23	0.15	0.28	0.23	"			
4	6.9	" 15	Jan. 7	Jan. 12	5	Jan. 10	0.663	645	0.079	0.41	0.35		0.56	0.43	"	Thin.		
6	5.7	" 24	" 19	" 19	5	" 18	0.687	574	0.186 0.19	0.48		0.35	0.59	0.47*	"	Very thin.		
7	10	Jan. 12	" 22	" 27	5	" 26	0.679	545	0.063	0.26	0.32	0.23	0.27	0.26	"			

\* Muscles, particularly sartorius, were removed along with small amount of adhering fat. Because of rapid breakdown of glycogen muscle following removal from body (Macleod and Simpson) it was not thought advisable to remove the fat before treating with KOH.

animals guillotined on the 5th day the liver glycogen percentages were 0.077, 0.18, and 0.063, or in total amounts, calculated as above, 0.265, 0.513, and 0.315 gm. respectively. If the difference between the averages of each group (*viz.* 1.170 and 0.364) = 0.806 gm. of glycogen, it could have been excreted from the liver as sugar between the 3rd and 5th days.

The muscles contained almost as high a percentage of glycogen after 5 days as after 3 days. Since glycogen disappears after death from the muscles of warm blooded animals with extreme rapidity, and since the muscles of the right leg were dissected out first, we will consider these alone. The average percentages are:

After 3 days: 0.44, 0.47, 0.39, 0.46 per cent; average 0.44.

“ 5 “ 0.23, 0.38, 0.48, 0.29 “ “ “ 0.34.

Suppose the muscles constitute 40 per cent of the body weight, then these percentages in each case correspond in total glycogen to:

After 3 days: 12.7, 11.7, 16.6 gm.; average 13.7.

“ 5 “ (4.97), 10.5, 10.9, 11.6 gm.; average 11.0.

so that between the 3rd and 5th day of fasting, 2.7 gm. of muscle glycogen on an average might have been excreted as sugar, if for the moment we suppose that such a transformation were possible. Taking into consideration the glycogen which disappears from both liver and muscles between the 3rd and 5th days, a total of  $0.8 + 2.7 = 3.5$  gm. of glucose might be excreted (average body weight, 8.6 kilos).

Let us see to what extent the excretion of this amount of glucose might alter the D:N ratio in Dog C. When the animal weighed 8.6 kilos 52.7 gm. of glucose and 9.71 gm. of nitrogen were excreted during the above interval (3rd to 5th day), giving a D:N ratio of 5.42; after subtraction of the glycogen which disappeared from the liver and muscles this becomes reduced only to 5.08. A similar correction (allowing for body weight) applied to the results of Dog F when it weighed 5.9 kilos reduces the D:N ratio from 3.76 to 3.49.

It is impossible, therefore, to account for the high D:N ratios often observed beyond the 3rd day after removal of insulin and food, as dependent on excretion of stored carbohydrate, even when as in the above calculations we make the unwarranted

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assumption that muscle glycogen may contribute to the glucose excreted in the urine. It may furthermore be pointed out that the total amount of glycogen in the muscles 3 days after pancreatectomy, *viz.*  $\left(\frac{13.7}{8.6}\right) = 1.6$  gm. per kilo of body weight, would suffice to maintain the energy requirements for not more than 2 hours. Thus, the average  $O_2$  consumption for all the animals was 645 cc. per kilo and hour, and 1.6 gm. of carbohydrate would require about 1200 cc. of  $O_2$  to oxidize it. If we assume that the fuel used by the muscles is exclusively their own glycogen and that this is replenished by gluconeogenesis going on in the liver, the foregoing results also show that protein cannot be the only material used in the process. Thus the D:N ratio even after every possible allowance for glycogen present in liver and muscle is made is often found to be greater not only than 2.8 which Minkowski supposed must represent the protein ratio, but also than 3.65 which Lusk found to be its level in animals under the influence of phlorhizin.

We may, therefore, conclude that some of the glucose which is excreted after withdrawal of insulin in well nourished depancreatized dogs is derived from fat.

Although the D:N ratios are not as a rule corrected for glucose coming from glycerol, it is of interest to see to what level such correction would bring the ratio already corrected for glycogen, *viz.* 5.08.

The average  $O_2$  consumption per kilo of body weight and per hour for all dogs is 645 cc., which with a R.Q. of 0.70 equals 3.02 calories.

The average N excretion of Dog C (weight 8.6 kilos) between the 3rd and 5th days was 0.023 gm. per kilo and hour.

Since 1 gm. N = 26.51 calories (Lusk),

0.023 gm. = 0.61 "

Calories derived from fat = 2.410.

Since 1 gm. fat yields 9.4 calories,

2.41 calories = 0.256 gm. fat, or

0.0256 " glycerol.

The average glucose excretion per kilo and hour was 0.117 gm. or after deducting glucose derived from glycerol 0.092 gm., so that the D:N ratio becomes  $\frac{0.082}{0.023} = 4.0$  instead of 5.08.

Even when every possible allowance is made for sugar formation from glycogen and the glycerol fraction of fat, the D:N ratio in well nourished depancreatized dogs may be decidedly higher than 3.65, which according to present day beliefs is indisputable evidence that sugar is derived from fatty acid. That these higher ratios should be observed most frequently in animals that are richly fed may depend on the fact that there is usually considerable deposition of fat in fat depots under these conditions. It is possible that this fat is more readily available for sugar formation than is the tissue fat, which makes up the greater proportion of the total fat of the body in ill nourished animals.

*Excretion of Ketone Bodies.*—Since previous investigations in this laboratory had shown that the percentage of ketone bodies in the blood of depancreatized dogs after withdrawal of food and insulin was higher in well nourished as compared with lean animals, it was expected that corresponding differences would reveal themselves in the total amounts excreted by the urine of the same animal in varying states of nutrition. The present results (see Tables I to III) do not show this difference. On the contrary, when reduced to per kilo of body weight and hour, the excretion of total ketone bodies by the same animal was, if anything, somewhat greater when the body weight was low than when it was high. The ratios between acetone + acetoacetic acid and  $\beta$ -hydroxybutyric acid fractions were also not very different in the two conditions, although in all cases they were lower (*i.e.* hydroxybutyric acid relatively greater) when the animal was poorly nourished. These ratios were as follows:

*Ratios of Acetone + Acetoacetic Acid to  $\beta$ -Hydroxybutyric Acid.*

Dog F, 1 : 3.15 when weight was 5.9 kilos and 1 : 4.16 when weight was 4.8 kilos.

Dog C, 1 : 1.7 when weight was 8.6 kilos and 1 : 3.2 when weight was 6.36 kilos.

Dog T (fat), 1 : 1.38 when weight was 11.36 kilos.

“ D (lean), 1 : 1.20 “ “ “ 5.6 “

Whether this relatively greater excretion of  $\beta$ -hydroxybutyric acid in less well nourished animals is significant we cannot say. Several workers have recorded observations on the ratio of these two acids in the urine of diabetic patients. Kennaway (1914) found that from 70 to 80 per cent of the total ketone bodies

excreted was in the form of  $\beta$ -hydroxybutyric acid. This corresponds to a ratio of 1:3. In two of his four patients Hurlley (1915-16) observed ratios of about 1:3; the other two showed considerable variation from 1:3 to 1:1. The conditions under which these results were obtained are not strictly comparable to those reported here, for in the human patients the acetone bodies are derived, in part at least, from the ingested food, whereas in the fasting diabetic dog, they represent the fat and protein broken down from the animal's own tissues. Reports of the amount of ketone bodies excreted by depancreatized dogs are difficult to find in the literature, although there are frequent references to work of a qualitative nature. Allen and Wishart (1923) found in a well nourished 20 kilo dog treated with phlorhizin that 4 gm. of total acetone bodies were excreted on the 5th day. This corresponds with the amounts found in the present research on depancreatized animals, but the authors remark that the above result was the highest one obtained in a considerable number of animals and that much lower values are the rule after pancreatectomy. Our results show on the contrary that the total ketone body excretion may be considerably higher, *viz.* 0.28 gm. per 24 hours and kilo (5.6 gm.) and indeed that it is not far removed from the average amounts excreted by human diabetic patients (*cf.* Joslin, 1923).

Although nothing of significance with regard to their derivation can apparently be learned by comparison of the excretion of ketone bodies in depancreatized dogs in different states of nutrition, important facts reveal themselves when their excretion in relationship to that of sugar and nitrogen is followed from day to day. This is evident from a study of the data of Tables I, II, and III.

A remarkable uniformity is evident in the different tables, and the following conclusions may be drawn from the results.

1. The excretion of glucose falls until the 3rd day after which in most cases it rises for about  $1\frac{1}{2}$  or 2 days. Following this there is a decrease in the glucose excretion.

2. The excretion of nitrogen rises steadily from the 2nd day until the 4th or 5th, when it either becomes steady (Dogs C<sub>1</sub> and F<sub>1</sub>) or tends to decline (Dogs C<sub>2</sub>, F<sub>2</sub>, T). Sometimes, as in Dog D, the nitrogen excretion remains fairly constant from day to day.

3. Ketone bodies do not as a rule make their appearance in the urine in measurable quantities until the fourth or fifth 12 hour period after removal of insulin, and there is no constant difference in the time of appearance according to whether the animal was well or poorly nourished.

4. After it has once set in the excretion of ketone bodies rises rapidly from period to period until the seventh or eighth (4th or 5th day after removal of insulin), when it declines again and may fall almost to the vanishing point (Dog F<sub>2</sub>) by the ninth period. In a general way this excretion runs parallel with that of glucose, this relationship being particularly well marked in Dog F<sub>2</sub> and Dog C<sub>1</sub> and C<sub>2</sub>.

A similar parallelism between the glucose and ketone excretion has been observed by other workers. Baer and Blum (1923) showed that the administration of leucine to diabetic patients led to an increased excretion of both glucose and ketone bodies in the urine. Burn and Marks (1926) perfused the isolated liver of a dog and found that the formation of glucose was always associated with the presence of increased amounts of ketone bodies.

It is significant that the decline in ketone excretion is not accompanied by an increase in nitrogen, but rather the reverse. This may depend on the fact that some of the ketone bodies are derived from amino acids.

5. The  $\beta$ -hydroxybutyric acid may rise more rapidly than the acetoacetic acid plus acetone, but the latter tends to approach more nearly to the former when the animal is well nourished. Indeed the acetoacetic acid plus acetone fraction may be nearly as great as that of  $\beta$ -hydroxybutyric acid. This relative increase of acetone plus the acetoacetic acid fraction in well nourished animals, which has already been referred to, is clearly seen by comparison of data in the table for Dog C (C<sub>1</sub> and C<sub>2</sub>) and is also evident in those for Dog F (F<sub>1</sub> and F<sub>2</sub>). Similar changes in the relationship of  $\beta$ -hydroxybutyric acid to the other ketone bodies were also found by Chaikoff *et al.* when the percentage in the blood was examined, there being relatively less  $\beta$ -hydroxybutyric acid in fat, as compared with thin animals.

It has not, therefore, been possible in the present investigation to show that the excretion of total ketone bodies is any greater when the animal is fattened than when it is thin. This absence



of any difference may merely depend on the fact that in both nutritional states there was abundance of available fat to be mobilized after the stored glycogen had been used up, and it may indicate that the factor which determines what amount of ketone bodies will result as by-products in the conversion of fatty acid into sugar is one that is entirely independent of the available fat.

It has been stated that the time of appearance of ketone bodies in the urine corresponds to that at which glycogen disappears from the liver. Our results show that there still remains about 0.3 per cent of glycogen in this viscus in animals killed on the 3rd day after withdrawal of insulin and that over 0.1 per cent may still be present on the 5th day, by which time in all our observations the excretion of ketone bodies has become considerable. In a general way one may say that ketonuria sets in at the time when the glycogen stores of the liver have almost become exhausted, but there is considerable overlapping. We are doubtful whether all traces of glycogen ever disappear from the liver following pancreatectomy; at least we have always found *some*, provided care was taken to remove the viscus for analysis immediately after decapitation of the animal. It is also of interest to note that the respiratory quotient attains the diabetic level of about 0.67 at least by the 3rd day after withdrawal of insulin when ketonuria has become a prominent symptom. There is, therefore, no doubt that ketonuria becomes decided at about the time when glycogen almost disappears from the liver and when the R.Q. falls to the diabetic level.

In contrast to that of the liver, the glycogen of the muscles remains fairly constant in amount at least after the 3rd day of fasting (*cf.* Table IV). Assuming that, as in normal animals, this glycogen is constantly being used in the production of energy in the muscles, then it is evident that new formation of muscle glycogen must constantly be occurring, the raw material for which is the blood sugar manufactured in the liver by a process of glycconeogenesis.

From the result of the present investigation it is difficult to see how insulin can be essential for the formation of glycogen in the muscles. It is possible that glycogen formation in the liver is an obligatory preliminary step to this latter process when no

insulin is available, and that gluconeogenesis becomes excessive whenever the percentage of glycogen in the liver falls below a certain level, which may be about 0.3 per cent.

#### CONCLUSIONS.

The present observations were made on depancreatized dogs brought into varying states of bodily nutrition by treatment with insulin and control of the diet.

1. The D:N ratios of the same depancreatized dog after withdrawal of food and insulin vary according to the nutritive condition of the animal.

2. Between the 3rd and 5th days after withdrawal of insulin, the D:N ratio may be considerably above the value found in phlorhizinized dogs.

3. About 0.3 per cent of glycogen may remain in the liver, and 0.45 per cent in the muscles on the 3rd day after withdrawal of insulin, and by this time the R.Q. is between 0.66 and 0.70.

4. After the 5th day the percentages become reduced to 0.06 and 0.35 respectively, and after making allowance in the D:N ratio for the glucose thus derived, ratios of over 5 were obtained.

5. Ketone bodies in measurable amounts appear in the urine in from 48 to 60 hours following withdrawal of insulin, there being no difference in this regard in thin as compared with well nourished animals.

6. The excretion rises to a maximum between the 4th and 5th days, after which it declines again. In a general way the curve of this excretion runs parallel with that of glucose.

7. The excretion of ketone bodies per kilo of body weight is much the same in depancreatized dogs as in diabetic patients.

8. The results of the investigation support the view that glucose is derived from fatty acid in the diabetic organism.

The author wishes to express his thanks to Professor J. J. R. Macleod under whose direction this research was carried out.

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## ALCOHOLIC CONTENT OF NORMAL PLACENTAL TISSUE.

BY WILLIAM D. McNALLY, H. C. EMBREE, AND C. A. RUST.

*(From the Chemical Laboratory, Cook County Coroner's Office, Chicago.)*

(Received for publication, May 4, 1927.)

A review of the literature shows that work has been done on the normal alcohol content of the blood and tissues of animals and fowls, blood and urine of human beings, with very little work on human tissues. Ford (1) was the first to report the presence of alcohol in blood, reporting 0.0009 per cent in fresh ox blood. Lenoble and Daniel (2) found normal human cerebrospinal fluid to contain 0.002 per cent alcohol. Nicloux (3) reports dog blood to contain 0.0018 per cent alcohol; Völtz and Dietrich (4) found in dog stomach 0.003 per cent, dog intestine 0.0023 per cent. Pringsheim (5) and Aoki (6) report the alcohol in rabbit blood 0.0018 to 0.003 per cent, rat muscle 0.0028 per cent, and rat liver 0.0020 per cent. Maignon (7) and Schweisheimer (8) found 0.0016 to 0.0036 per cent alcohol in human blood. Gettler (9) reports 0.0006 to 0.0023 per cent in human brain.

This paper is a report of work done with about 100 human placentas (see Table I). The placentas were secured from the Obstetrical Department of the Cook County Hospital and analysis was begun within a few hours of the expulsion of the placenta.

In accordance with the method developed in this laboratory, two or three placentas (enough to make 1500 to 2000 gm.) were weighed, cut fine in a food chopper, acidified with tartaric acid, and distilled in a current of steam, until 1 cc. of distillate was collected for each gm. of the material used. The first distillate was slightly cloudy, containing fatty acids, alcohol, and acetone. The distillate was redistilled repeatedly discarding a 40 per cent residue at each distillation until the distillate reached 100 cc. After the first 40 per cent was discarded the distillate failed to show the presence of acetone when the nitroprusside test (Legal's)

was made. To this distillate were added 5 cc. of 10 per cent sodium hydroxide solution and 3 cc. of a 10 per cent silver nitrate solution to oxidize the volatile aldehydes and saponify any of the fats and also fix the fatty acids present. The mixture was re-distilled and 50 per cent of the distillate collected each time until it was reduced to a volume of about 10 cc. According to Jansch (10) this distillate contained only methyl and ethyl alcohols. The final distillate was weighed in a pycnometer and the refractive index taken with a Zeiss immersion refractometer; the relative quantities of methyl and ethyl alcohol were determined by consulting the tables of Wagner (11), Windish (12), and von Fellenberg (13).

TABLE I.  
*Alcoholic Content of Normal Placentas.*

Experi- ment No.	Alcohol.	Experi- ment No.	Alcohol.	Experi- ment No.	Alcohol.	Experi- ment No.	Alcohol.
	<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>
1	0.0043	8	0.0009	15	0.0018	22	0.0020
2	0.0031	9	0.0052	16	0.0010	23	0.0035
3	0.0046	10	0.0031	17	0.0011	24	0.0040
4	0.0025	11	0.0020	18	0.0013	25	0.0031
5	0.0022	12	0.0008	19	0.0031	26	0.0011
6	0.0044	13	0.0025	20	0.0042	27	0.0016
7	0.0040	14	0.0020	21	0.0052	28	0.0037

The ethyl alcohol was identified by the iodoform test, benzoyl chloride test, and the burning test. The quantity of ethyl alcohol was determined by a modified Nicloux method as follows: 5 cc. of the distillate were placed in a 30 cc. Erlenmeyer flask with a graduated pipette. Two burettes were provided; one was filled with concentrated sulfuric acid, and the other, which was graduated to 0.05 cc., was filled with standard potassium dichromate solution, 19 gm. per liter. From the sulfuric acid burette 3 cc. of concentrated sulfuric acid are run into the flask, and the dichromate is then added a little at a time, and the flask is shaken after each addition until the blue-green which appears at first gives way to a yellow-green. The flask is then placed in water at 100°C. for 2 minutes, and more dichromate added if necessary, until the yellow-green tint persists after 2 minutes of warming. A second 5 cc. portion of the distillate is pipetted into a clean

flask for a check titration; this time a quantity of dichromate is added which is slightly less than the quantity first added. This serves as a check and also to eliminate the possibility of loss of alcohol in the heating process. 1 cc. of the dichromate solution is equal to 0.10 per cent alcohol in the distillate when 5 cc. of the distillate are used for the titration.

The following tests for methyl alcohol were negative: the morphine sulfate test, the resorcinol test (after oxidizing with heated copper spiral), and a modified U.S.P. potassium permanganate test as follows: Place 5 cc. of a 1 per cent solution of potassium permanganate in a test-tube, add 0.1 cc. of the specimen and 0.2 cc. of concentrated sulfuric acid; thoroughly mix the solution by shaking and allow it to stand 3 or 4 minutes. Now add 1 cc. of an 8 per cent solution of oxalic acid, shake, and allow to stand until the mixture is decolorized; then add 1 cc. of concentrated sulfuric acid and 5 cc. of fuchsin bisulfite solution; mix and allow to stand. The presence of methyl alcohol is indicated by the reddish violet color which gradually develops. The intensity of the reaction depends on the quantity of methyl alcohol present. The color appears in 5 minutes when present in a quantity as small as 0.10 per cent. In weaker solution the color may appear blue at first, but when observed through the long axis of the test-tube the violet color stands out permanently, the maximum color developing in about 45 minutes. A solution of a decolorized fuchsin (Schiff's reagent) is a general aldehydic reagent with neutral or slightly acid solutions, but in a solution containing 15 to 20 per cent by weight of the concentrated sulfuric acid the color is restored only by formaldehyde. This method we found to be more delicate than the other methods for detecting methyl alcohol. Schaffer (personal communication) found that the most sensitive fuchsin bisulfite solution should be prepared as follows: Dissolve 0.5 gm. of fuchsin (basic) in 200 cc. of distilled water; add an aqueous solution of sulfur dioxide, the quantity corresponding to 1 gm. of sulfur dioxide gas; allow to stand until the solution assumes a light amber color (this requires about 1 hour). Make up to 400 cc. with distilled water; mix well and keep in a cool place (ice box).

After a number of distillations was made it was found that time could be saved by distillation through a special fractionating

tower at a constant temperature of 97°C. until the amount reached about 100 cc., and then treating as before.

The fractionating tower used is an ordinary fractionating cylindrical tube into which indentations have been made, a continuation of a plain cylindrical tube to which a side arm with a distillate outlet is attached. There is an opening at the top of the side arm so as to allow the insertion of a thermometer for recording the temperature of the distillate coming over. A flexible, sealed water-cooled reflux is inserted into the top of the plain section of the column. By raising or lowering this reflux in the plain section, the composition of the fraction coming over can be controlled.

#### DISCUSSION.

Gettler's (9) statement, "No faith can be placed in any of the figures [in previous articles on normal alcohol] because methods used were not specific for ethyl alcohol" is only partially true. The figures of the early investigators correspond closely with Gettler's figures and with our own and we believe that they had ethyl alcohol. In our method acetone and other substances responding to the iodoform reaction and burning test are eliminated. The figures quoted by Gettler of Landsburg, Maignon, Pringsheim, and Schweisheimer should have one more cipher placed in front of the digit reading 0.0028, 0.0016, 0.0044, and 0.00368 per cent respectively.

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# THE CERULEOMOLYBDATE ESTIMATION OF PHOSPHATE-PHOSPHORUS.\*

BY B. E. GILBERT AND J. B. SMITH.

(From the Rhode Island Agricultural Experiment Station, Kingston.)

(Received for publication, May 9, 1927.)

In 1920 Denigés<sup>1</sup> introduced the ceruleomolybdate method for the determination of the phosphate ion in biological materials. Since that time this method has been used by Florentin<sup>2</sup> with river waters, by Atkins,<sup>3</sup> Parker,<sup>4</sup> and others for a rapid method of determining the soluble phosphate in soils, and by Gilbert<sup>5</sup> for the estimation of phosphate in plant solutions. These varied uses of the method have all been concerned with solutions which were by nature not far removed from pH 7 in reaction. When it was endeavored to use the method for the estimation of phosphate in extracts of soil made with strong acids, a distinctly inhibiting effect on color formation was noted. In this paper such effects are discussed and further evidence introduced to show the sensitivity of the color complex to acids, bases, and salts.

## *Description of Method.*

### *Solutions.*

**Reagent A.**—A mixture of 100 cc. of 10 per cent ammonium molybdate with 300 cc. of 50 per cent (by volume) sulfuric acid. This reagent must be stored in the dark.

**Reagent B.**—Stannous chloride solution prepared by using 0.25 gm. of mossy tin metal, 3 drops of 4 per cent copper sulfate solu-

\* Contribution No. 349 of the Rhode Island Agricultural Experiment Station.

<sup>1</sup> Denigés, G., *Compt. rend. Soc. biol.*, 1920, lxxxiv, 875.

<sup>2</sup> Florentin, D., *Ann. chim. anal. et chim. appl.*, 1921, iii, 295.

<sup>3</sup> Atkins, W. R. G., *J. Agric. Sc.*, 1924, xiv, 192.

<sup>4</sup> Parker, F. W., *Soil Sc.*, 1925, xx, 149.

<sup>5</sup> Gilbert, B. E., *Plant Physiol.*, 1926, i, 191.



tion, and 2 cc. of concentrated hydrochloric acid. The reaction is hastened by warming and finally the volume is made up to 25 cc.

*Standard Phosphate-Phosphorus Solution.*—This is prepared by dissolving  $\text{KH}_2\text{PO}_4$  in distilled water and having an analyzed strength of 0.5 parts per million of phosphate-phosphorus.

*Solutions of Different Normalities of Single Acids, Bases, and Salts.*—The acids used were hydrochloric, sulfuric, nitric, acetic, oxalic, citric, and boric. Sodium and ammonium hydroxides represented bases and the salts investigated were sodium nitrate, sodium borate, potassium sulfate, ammonium nitrate, sodium oxalate, ammonium oxalate, and sodium acetate.

#### *Procedure.*

In order to determine the effects of acids, bases, and salts upon the color formation, 20 cc. volumes of the standard solution were placed in 100 cc. volumetric flasks. To five of six such solutions varying volumes of an acid, base, or salt solution of known normality were added. The sixth solution was used as a standard. All the solutions were next diluted to 95 cc. with distilled water. Then, following the procedure as outlined by Atkins<sup>2</sup> 2 cc. of Reagent A were added and each solution was mixed by shaking. Finally, 6 drops of Reagent B were added and each solution, including the standard, was made up to volume and carefully mixed. The color was allowed to develop for 10 minutes, at the end of which time each solution was compared with the standard by means of a Spencer-Duboscq colorimeter. From the known concentration of the standard the amount of phosphate-phosphorus as shown by the color in each solution was calculated.

#### EXPERIMENTAL.

Following the method, as outlined above, the results obtained are shown in tabular form. The normalities which are given indicate the concentration of acid, base, or salt at final dilution. The pH acidity figures were either calculated or determined by use of the quinhydrone electrode. Neither normalities nor pH figures should be considered extremely definite since the problem did not seem to warrant extreme accuracy. The color readings were made with a Spencer-Duboscq colorimeter.

*Strong Acids.*

From a consideration of Table I it will be seen that on addition of hydrochloric acid with concentrations greater than 0.02 N, and acidities stronger than 1.72 pH, the amounts of phosphate as measured by color decreased. Similar results were obtained with sulfuric and nitric acids. Thus, strong acids, present in moderate concentrations, interfere with color formation.

TABLE I.

*Effects of Several Normalities of Acids on Phosphate Determinations.*

Normality.*	Phosphate-phosphorus supplied.†	Phosphate-phosphorus found (parts per million).									
		Oxalic acid.		Citric acid.		Hydrochloric acid.		Acetic acid.		Boric acid.	
		pH*		pH*		pH*		pH*		pH*	
0.001	0.100	3.05	0.083	3.50	0.094						
0.002	0.100	2.83	0.060	3.46	0.086						
0.003	0.100	2.70	0.000	3.21	0.069						
0.004	0.100			3.10	0.044						
0.020	0.100					1.72	0.100				
0.050	0.100					1.34	0.092			5.28	0.100
0.125	0.100					0.94	0.048	2.82	0.100	5.08	0.100
0.320	0.100							2.62	0.091		
0.660	0.100							2.47	0.085		

\* Normality and pH of acid at final dilution.

† Measured in parts per million of phosphate-phosphorus supplied in solution.

*Weak Acids.*

A corresponding effect was found with weak acids (Table I) although the story is not so definite. Citric and oxalic acids affect color formation in much lower concentrations than do acetic and boric acids. Phenol and boric acid had no effect at the concentrations used.

Acetic acid, boric acid, and phenol had no inhibiting effect on color formation at a strength of 0.125 N. Normal acetic acid was found to inhibit color formation.

*Bases.*

When different normalities of sodium hydroxide and ammonium hydroxide were used, the general result was a deepening in the

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intensity of the color for all concentrations greater than 0.0025 N (Table II). At pH 11.38 the strong base had no effect while the weaker base did not intensify the color at pH 10.31.

### *Salts.*

From the data given in Table II, it is seen that sodium nitrate had no effect on color formation in concentrations from 0.001 N to 0.125 N. Sodium borate, potassium sulfate, and ammonium nitrate also did not inhibit color formation at these concentra-

TABLE II.

*Effects of Several Normalities of Bases and Certain Salts on Phosphate Determinations.*

Normality.*	Phosphate-phosphorus supplied.†	Phosphate-phosphorus found (parts per million).							
		Ammonium hydroxide.		Sodium hydroxide.		Sodium oxalate.	Ammonium oxalate.	Sodium acetate.	Sodium nitrate.
		pH*		pH*					
0.0001	0.100					0.100	0.100		
0.0003	0.100					0.096	0.096		
0.0005	0.100	9.96	0.100			0.088			
0.0015	0.100	10.20	0.100						
0.0025	0.100	10.31	0.100	11.38	0.100				
0.010	0.100			11.90	0.106				
0.025	0.100	10.81	0.111	12.30	0.120			0.100	0.100
0.075	0.100	11.05	0.118	12.80	0.116			0.125	0.100
0.125	0.100	11.16	0.230					0.207	0.100

\* Normality and pH of base or salt at final dilution.

† Measured in parts per million of phosphate-phosphorus supplied in solution.

tions. Sodium oxalate and ammonium oxalate caused pronounced inhibition and even 0.0002 N brought about a reduction in color. Sodium acetate of 0.04 N increased the intensity of the color.

### *Some Properties of the Color Complex.*

In view of the reducing proclivities of the oxalate ion, portions of the standard colored solution were subjected to reducing conditions. Zinc dust when added to the colored solution, which contained excess hydrochloric acid from Reagent B, generated

hydrogen gas and the color was quickly destroyed. When sulfur dioxide gas was bubbled through the colored solution the color gradually decreased in intensity. The possibility of the reduction of the color may aid in explaining the extreme inhibition brought about by the oxalate ion.

Some few attempts were made to determine the physical state of the color complex. After 19 hours no dialysis was obtained with a collodion membrane, care having been taken to have similar osmotic concentrations on both sides of the membrane. When filter paper impregnated with collodion was used, no color came through even when the system was connected to a vacuum. On heating the colored solution the color quickly decreased in intensity and this fading was definitely accelerated by the addition of a few drops of a 1 per cent solution of aluminum sulfate. In all cases the fading of the color is accompanied by the production of a turbidity, which invariably develops as the color fades on standing in the cold.

The colored solution was subjected to conditions of cataphoresis but the results were negative, no movement of color showing after a period of 12 hours.<sup>6</sup>

During the first stages of the investigation some correlation between the inhibitory effect of acids and the pH of the solutions seemed to be shown. This, however, failed to appear when oxalic and acetic acids and sodium acetate were investigated. From the failure of the color to dialyze, the complex may be assumed to exist as a colloid and if such is the case weak acids and salts of weak acids would not be expected to have the same coagulation effects upon a suspension. More work, however, is necessary to establish definitely the physical state of the color complex.

#### DISCUSSION.

From the foregoing data it is apparent that the effects of acids, bases, and salts on the formation of the color complex are sufficiently great to limit, to a narrow range, the usefulness of the method as heretofore described. Particularly is the practicability

<sup>6</sup> The authors are indebted to Prof. Lester Coggins of the Physics Department of the Rhode Island State College for his advice and for use of equipment.

of the method restricted when it is desired to estimate phosphate in the presence of strong or weak acids of relatively great concentration as suggested by Parker.

In an endeavor to overcome this difficulty (the acidity of the unknown solution being known) the feasibility of estimating the strength of an unknown concentration of phosphate-phosphorus in acid solution by comparison with a standard identical in acid concentration, was considered. Solutions were prepared by mixing known volumes of a standard phosphate solution with a constant volume of an acid, the final acid normality at time of color development being equal in all cases. In this manner varying amounts of phosphate were estimated in the presence of

TABLE III.

*Estimation of Varying Amounts of Phosphate-Phosphorus in Presence of Constant Amount of an Acid.*

Results are measured in parts per million of phosphate-phosphorus supplied in solution.

0.1 N hydrochloric acid.		0.1 N nitric acid.		0.1 N sulfuric acid.		N acetic acid.	
Theoreti-cal.	Found.	Theoreti-cal.	Found.	Theoreti-cal.	Found.	Theoreti-cal.	Found.
0.25	0.28	0.38	0.42	0.38	0.41	0.62	0.62
0.37	0.37	0.50	0.50	0.50	0.51	0.37	0.38
0.62	0.58	0.63	0.59	0.63	0.57	0.25	0.26

0.1 N hydrochloric acid, 0.1 N sulfuric acid, 0.1 N nitric acid, and N acetic acid. These normalities are those obtaining in the solutions at time of the development of color and were chosen since they had proved, from the data in Tables I and II, to give definite color inhibition. The results of these estimations are given in Table III.

From a consideration of these results it would seem feasible to estimate phosphate in the presence of acids, providing the comparison is made with a color standard containing the same acid in equal concentration.

#### SUMMARY.

Evidence is given to prove that the ceruleomolybdate estimation of the phosphate ion is limited in application. Strong and

weak acids caused serious inhibition of the color formation at concentrations depending upon the particular acid. Sodium hydroxide and ammonium hydroxide greatly intensified the color, and certain salts inhibited while others intensified it.

A method is suggested for use with strong acids and acetic acid.

Some properties of the color complex are discussed which show its susceptibility to reducing conditions and point to its colloidal nature.



## THE BLOOD PEPTIDE NITROGEN IN ARTERIAL HYPERTENSION.

By HENRY JACKSON, JR., DAVID W. SHERWOOD, AND  
OLIVE J. MOORE.

*(From the Thorndike Memorial Laboratory, Boston City Hospital, and the  
Department of Medicine, Harvard Medical School, Boston.)*

(Received for publication, June 6, 1927.)

Hülse and Straus (1) in 1924 concluded that the blood plasma of patients with arterial hypertension contained notable amounts of polypeptide nitrogen, and on the basis of some animal experimentation reported in the same paper they put forth the hypothesis that nephritic hypertension is due to the presence in the blood of these partially split protein products.

Blau in 1923 (2) showed that normal blood plasma contained up to 3 mg. of peptide nitrogen per 100 ml., and in this Hülse concurs. The latter author maintains, however, that the peptide nitrogen may rise in hypertension to as much as 30 mg. per 100 ml. when estimated by the formol titration method.

To check the results of Hülse a method was devised by which the peptide nitrogen of the blood could be determined with reasonable accuracy in small samples of blood.

10 ml. of clear plasma were diluted with 10 ml. of distilled water and treated with 20 ml. of a 5 per cent trichloroacetic acid solution. The mixture was thoroughly shaken and allowed to stand for  $\frac{1}{2}$  hour. Water-clear filtrates were always obtained and in no filtrate examined did we find evidence of coagulable protein. For the determination of the free amino acids 5 ml. of this filtrate were used as in Folin's colorimetric determination. Care must be taken to neutralize carefully the trichloroacetic acid before adding the amino acid reagent.

For the determination of the peptide nitrogen 20 ml. of the trichloroacetic acid filtrate were treated with 20 ml. of concentrated HCl and the mixture was heated in a water bath for from



8 to 12 hours. It was then quantitatively transferred to an evaporating dish and evaporated over a steam bath to dryness. Exactly 20 ml. of distilled water were added and while still slightly acid the solution was twice extracted with permutit to remove any ammonia which might have been formed from urea by acid hydrolysis. The amino acids were again determined in a suitable sample of the clear filtrate from the permutit extraction. The difference between the figures given by the two procedures gives the polypeptide nitrogen. The following protocols show the accuracy and limitations of the method. Kahlbaum's glycine was used as the added amino acid and Witte's peptone was used as a source of polypeptide nitrogen. Carefully filtered solutions of

TABLE I.

Results are expressed as mg. per 100 ml.

Plasma amino N.	Amino N added.	Amino N found.	Recovery.
			<i>per cent</i>
5.3	8.2	13.3	97
5.0	14.3	18.0	97
Plasma amino N.	Peptide N added.	Total amino N found.	Recovery.
			<i>per cent</i>
4.3	8.2	8.9	56
5.1	14.0	17.0	80
5.1	20.3	24.3	94
5.2	150.0	151.4	97

Witte's peptone were used and the total nitrogen of the filtrate was taken to be the total polypeptide nitrogen. The small amounts of free amino acids were disregarded. See Table I.

It will be seen that recovery of free amino acids by this method is entirely satisfactory; that recovery of peptide N in low concentrations was reasonably good (56 per cent) and in high concentrations excellent (80 to 97 per cent). It may be said here that small amounts of peptide nitrogen (1 to 4 mg. per 100 ml.) cannot be accurately determined by this, or, we believe, by any other existing method for blood.

By use of the method outlined above, a series of cases was studied. 50 cases in all were examined. Thirty-four had hypertension. On analysis of the figures obtained we find:

1. Cases which had a normal blood pressure showed an average peptide nitrogen of 0.8 mg. per 100 ml. with extremes of 0.0 and 5.8 mg. per 100 ml. Only three of these cases had a peptide nitrogen of over 1 mg. per 100 ml. One case of severe bichloride poisoning had a peptide N of 5.8, the highest figure encountered.

2. Cases of hypertension showed an average peptide N of 0.7 mg. with extremes of 0.0 and 5.2. Of these hypertensive cases (thirty-four) six had a peptide nitrogen of over 1 mg. per 100 ml., and twenty-eight were under 1 mg. per 100 ml. The degree of hypertension varied from 160 mm. to 255 mm. with an average of 196 mm.

3. Of the nine cases with a peptide nitrogen of 1 mg. or over, six had hypertension and three showed a normal pressure.

4. Of the six cases of hypertension associated with nitrogen retention, three had a peptide nitrogen of over 1 mg. but two showed no peptide nitrogen by the method used.

#### CONCLUSION.

We have found no convincing evidence to show that the peptide nitrogen in hypertension rises sufficiently to be of etiological importance.

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# A STUDY OF THE STIMULATING EFFECT OF THE AMINO ACIDS ON SUGAR METABOLISM WITH RESPECT TO THEIR OPTICAL ACTIVITY.

BY W. E. BURGE, GEORGE C. WICKWIRE, A. M. ESTES, AND  
MAUDE WILLIAMS.

(From the Department of Physiology, University of Illinois, Urbana.)

(Received for publication, June 6, 1927.)

This investigation was begun originally to determine whether the amino acids have any effect on the rate of sugar metabolism. The work had not progressed very far before it was apparent that the optically active amino acids increased sugar metabolism whereas the inactive ones did not.

Lavoisier (1) found that the ingestion of food increased oxidation in the body. Rubner (2) showed that protein increased metabolism more than did fat or carbohydrate, and designated this as the specific dynamic action of protein. Lusk (3) found that the administration of the amino acids increased heat production, and since protein is normally presented to the tissues as amino acids, the stimulating effect of protein is in reality due to the amino acids. The fact that all of these amino acids, with one exception, glycine, are optically active may have some significance.

The amino acids used in this investigation were *dl*-leucine, *dl*-nor-leucine, *dl*-isoleucine, *dl*-valine, glycine, *dl*-alanine, *dl*-phenylalanine, *l*-leucine, *d*-isoleucine, *d*-glutamic acid, *l*-histidine, *l*-aspartic acid, aminoids, and the sugar, dextrose. The sugar determinations were made according to the method of Benedict. Because of the directness and simplicity of the experiments and to avoid the complexity encountered in the higher animals and man, with their different organs and internal secretions, the single celled animal (*Paramecium caudatum*) was used in these experiments.

These single celled animals were raised in great numbers on an infusion made of lake water and alfalfa. They were collected and washed free of debris with the use of a small centrifugalizing

machine. The centrifugalizing tubes were graduated in cc., so the organisms were measured as they were collected. Air was kept bubbling through the liquid containing the organisms at all times to insure an adequate supply of oxygen.

The following is the description of a typical experiment. 35 cc. of paramecia were collected, washed, and measured as described above. These were introduced into 1400 cc. of aerated lake water and to this were added 1.4 gm. of dextrose. After the sugar was dissolved, this 1400 cc. of paramecia-sugar preparation, while being thoroughly mixed by pouring from one vessel to another, was divided into 100 cc. batches. Each 100 cc. portion was introduced into a 200 cc. sedimentation glass and air was bubbled through it at a slow rate. 100 mg. of each of the amino acids were introduced into beakers and 10 cc. of water added and heated. A sufficient quantity of a saturated solution of sodium bicarbonate was added to form the sodium salts of the acids. Each of these 10 cc. solutions was added to 100 cc. of sugar-paramecia preparations and 10 cc. of water were added to the controls. Sugar determinations were made immediately and subsequently at intervals.

The results of the average of four series of experiments are given in Fig. 1. It will be seen that the controls used 27 per cent of the sugar in 8 hours, those to which the *dl*-phenylalanine was added 28 per cent, and so on for the other amino acids. By comparing the effect of these different amino acids on sugar utilization it will be seen that the optically inactive ones, namely *dl*-phenylalanine, *dl*-alanine, *dl*-leucine, glycine, *dl*-valine, *dl*-isoleucine, and *dl*-nor-leucine, had little effect on sugar metabolism, while the optically active ones, namely *d*-isoleucine, *l*-histidine, *l*-leucine, *d*-glutamic acid, *l*-aspartic acid, and aminoids, produced a large increase. It may be seen further in this chart that the aminoids used in the same concentration as were the amino acids, produced the greatest increase in sugar metabolism. These aminoids are a commercial product, biuret-free, with a total nitrogen of 10.93 per cent and amino nitrogen 75 per cent of the total nitrogen. We have carried out a great number of individual experiments similar to those described in the above series with fairly comparable results. If it is true that only the optically active amino acids stimulate metabolism, as these experiments

indicate, then it would seem to attach significance to the fact that practically all the naturally occurring amino acids and hence those normally presented to the tissues for use are optically active.

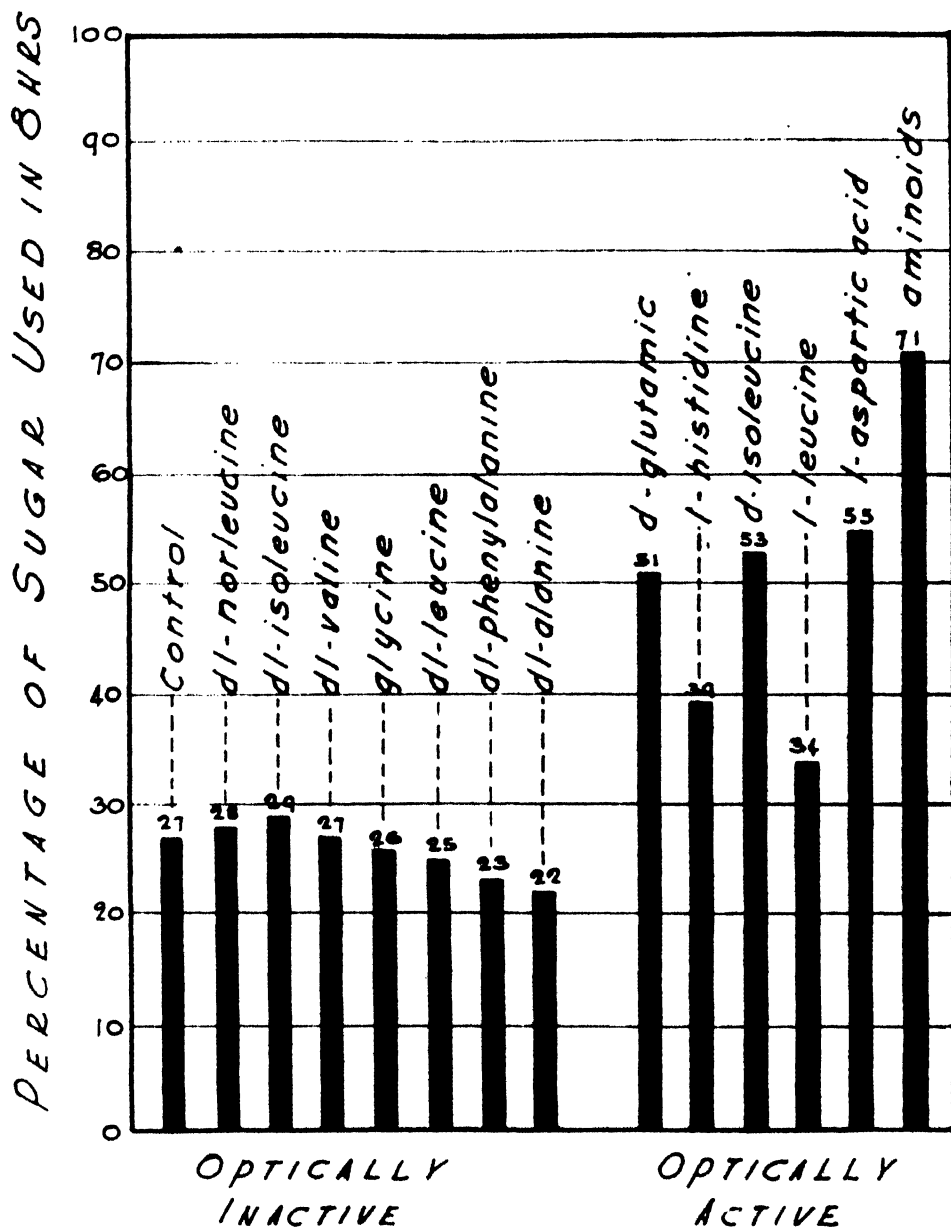


FIG. 1. Chart showing that the addition of optically inactive amino acids to sugar-paramecia preparations have no effect on the rate of sugar utilization, whereas the addition of optically active amino acids greatly increases the rate of sugar utilization.

Air was bubbled through sugar solutions of the same concentration without the paramecia as those used in this investigation for as much as 30 hours with practically no effect on the concentration of sugar. Hence the bubbling of air through the paramecia-sugar preparations in the experiments in this investigation had no effect *per se* on the sugar utilization. Paramecia-sugar preparation was made and the experiment permitted to run for 5 or 6 hours until approximately half of the sugar had been used. The preparation was then divided into two portions. One portion was centrifugalized and in this way the paramecia were removed, while the paramecia were left in the other. The sugar metabolism practically ceased in the portion from which the paramecia were removed, whereas in the portion in which the paramecia were permitted to remain all the sugar was used in the subsequent 4 or 5 hours. Such experiments are interpreted to mean that *Paramecium* and not extracellular enzymes or bacteria, etc., used the sugar in the experiments reported in this paper.

We have also studied the effect of the amino acids named in this paper on the sugar metabolism of the plant, *Spirogyra*, with essentially the same results that were obtained with the animal cell, *Paramecium*, the only difference being that *Paramecium* used the sugar much more rapidly than did *Spirogyra*. The question that presents itself is, Will these results hold true for man and the higher animals? This cannot be answered directly, but there are reasons for believing that they would, since sugar metabolism in *Paramecium*, *Spirogyra*, and man is alike in many respects. We (4) have found that *Paramecium* as well as *Spirogyra* utilized dextrose and levulose more rapidly than galactose, and that insulin increases the rate of this utilization just as is known to be the case with the higher animals and man.

#### SUMMARY.

1. The stimulating effect on sugar metabolism of seven optically inactive and five optically active amino acids was studied.
2. The optically active amino acids increased sugar metabolism whereas the optically inactive ones did not.
3. These observations suggest that optical activity may be the factor determining whether or not amino acids will stimulate metabolism.

4. The fact that practically all the amino acids normally used by the body are optically active would seem to lend further support to this view.

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## THE EFFECT OF INSULIN INJECTED INTO THE CEREBRO-SPINAL FLUID.

By J. V. SUPNIEWSKI, Y. ISHIKAWA, AND E. M. K. GEILING.

(From the Department of Pharmacology, Johns Hopkins Medical School, Baltimore.)

(Received for publication, May 23, 1927.)

It is a well established fact that readily diffusible substances pass freely from the cerebrospinal fluid into the venous circulation, while certain other compounds do not. The same principle holds true for drugs injected into the cerebrospinal fluid. For example, epinephrine injected directly into the subcerebellar region produces its characteristic physiological effects almost as rapidly as if injected directly into the blood stream. Postpituitary extract (puitritin of commerce) acts in a somewhat similar manner. Witte's peptone, on the other hand, when introduced into the subcerebellar cisterna does not escape from the cerebrospinal fluid (Dixon and Halliburton (1)).

Dixon (2) reported some experiments showing that insulin injected into the cerebrospinal canal has little hypoglycemic effect. From this he concluded that insulin passes out of the cerebrospinal fluid at a very slow rate. He also suggested that this indicates that the insulin molecule is a large one. These results aroused our interest and accordingly we conducted a number of experiments to determine the effect of insulin on the blood and the cerebrospinal fluid when injected either subcutaneously or directly into the cerebrospinal fluid by way of cerebellar puncture. We used dogs and rabbits anesthetized with isoamylethylbarbituric acid (amytal) (3) which causes the least change in blood sugar level of any of the commonly used hypnotics. The dose of amytal employed was 40 mg. per kilo of body weight introduced intravenously. Anesthesia followed in a few minutes.

In our first experiments samples of blood and cerebrospinal

TABLE I.

*Anesthetized (Amytal) Dogs Injected Subcutaneously with 3 Units of Insulin per Kilo of Body Weight.*

Dog No.	Time.	Sugar.	Cl	P
Blood.				
	hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	0	84		
	3	62		
2	0	95		
	3	44		
3	0	118		
	3	59		
4	0	96		
	3	45		
5	0	83		
	3	55		
6	0	84		
	3	46		
7	0	103	218	3.9
	1	103	254	2.9
	3	79	254	2.7
	5	57		2.8
Cerebrospinal fluid.				
1	0	62		
	3	55		
2	0	73		
	3	25		
3	0	84		
	3	30		
4	0	54		
	3	23		
5	0	53		
	3	28		
6	0	54		
	3	28		
7	0	76	422	2.2
	1	76	435	2.0
	3	62	402	1.2

fluid were taken about  $\frac{1}{2}$  hour after administration of the amytal and then 3 units of insulin<sup>1</sup> per kilo of body weight were injected subcutaneously. In all but one dog (No. 7) blood and cerebrospinal fluid were withdrawn at the end of 3 hours. The analyses are given in Table I, from which it will be clearly seen that the subcutaneous injection of insulin caused a decrease of sugar and phosphates in both the blood and the cerebrospinal fluid. Needless to mention here, these blood changes are well known (4) but our findings for the cerebrospinal fluid are in part new (5).

The subcutaneous injection of postpituitary liquid causes an increase of the blood and cerebrospinal fluid sugar and a decrease of lactic acid in both fluids. The data are given in Table II.

TABLE II.

*Anesthetized (Amytal) Dog Injected Subcutaneously with 1 Cc. of Pituitary\* Liquid.*

Time.	Blood.		Cerebrospinal fluid.	
	Sugar.	Lactic acid.	Sugar.	Lactic acid.
hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
0	98	36.0	66.0	31.8
3	116	27.3	82.0	28.1

\* Armour and Company.

In the main series of experiments the insulin in convulsive doses was introduced directly into the cerebellar cisterna. In the case of rabbits 3 units represented the total dose, while in dogs this was the dose per kilo of body weight. In each animal a subcutaneous injection of insulin in similar doses was given several days before or after the cerebellar cisternal injection. The results are given in Table III and show conclusively that after the intracerebellar injection the glucose in the cerebrospinal fluid and in the blood decreased very little, while the phosphates frequently showed a more pronounced fall. In a few cases where the cerebrospinal fluid contained blood, there was a considerable decrease of blood sugar, due without doubt to the mechanical escape of insulin into the circulation. In several instances there was a

<sup>1</sup> We are indebted to E. R. Squibb and Sons for kindly furnishing us with the insulin used in these experiments.

TABLE III.  
*Animals Injected with 3 Units of Insulin.*

Animal No.	Time.	Subcutaneous.		Into cerebellar cavum.	
		Sugar.	P	Sugar.	P
Blood.					
	hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Rabbit 1.	0	130		142	
	1.5	67		158	
	3.0	44		142	
	5.0	100		101	
Rabbit 2.	0	115	4.8	110	4.6
	1.5	43	2.1	120	3.8
	3.0	51	2.0	118	4.1
Rabbit 3.	0	100		81	
	1.5	45		77	
	3.0	55		52	
	5.0	89		52*	
Dog 1.	0	63	3.8	63	3.4
	1.5	38	2.2	155	4.1
	3.0	50	1.2	109	2.3
Dog 2.	0	92	3.8	63	3.4
	1.5	83	2.3	159	3.7
	3.0	58	1.5	61	3.3
Dog 3.	0	87	3.7	61	3.5
	1.5	47	2.5	132	4.8
	3.0	52	1.1	66	4.1
Dog 4.	0			100	
	1.5			122	
Dog 5.	0	100		96	5.6
	0.5	71		112	4.0
	1.0	55		110	4.0
	2.0	76		131	3.3
Dog 6.	0			96	5.3
	1.5			89	5.2
	3.0			53	3.9
	5.0			58	4.5

\* Blood in cerebrospinal fluid.

TABLE III—*Concluded.*

Animal No.	Time.	Subcutaneous.		Into cerebellar cavum.	
		Sugar.	P	Sugar.	P
Cerebrospinal fluid.					
	hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Dog 4.	0			85	
	1.5			149	
Dog 5.	0			80	1.25
	0.5			75	0.81
	1.0			98	1.5
Dog 6.	0			64	1.6
	1.5			66	1.4
	3.0			44*	3.3
	5.0				3.0

TABLE IV.

*Rabbits Injected Subcutaneously with 3 Clinical Units of Insulin Incubated at 37° 2½ Hours with 0.5 Cc. of Rabbit Cerebrospinal Fluid.*

Blood sugar.			
Time.	Rabbit 1.	Rabbit 2.	Rabbit 3.
<i>hrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
0	130	114	95
1.5	63	32	26
	Convulsions.	Convulsions.	Convulsions.

considerable rise in the sugar of the blood and the cerebrospinal fluid following the intracerebellar cisternal injection of insulin. At present we have no explanation to offer for this fact.

A control test was performed for the purpose of ascertaining whether traces of proteolytic enzymes found in the cerebrospinal fluid would be effective in decomposing some of the insulin, and whether if such a decomposition does occur it would afford an explanation for the failure of insulin to lower the blood sugar when introduced directly into the cerebellar cavum. Accordingly freshly drawn cerebrospinal fluid was mixed with insulin (9 clinical units with 15 cc. of cerebrospinal fluid) and incubated at 37° for 2½ hours and thereafter injected subcutaneously into three fasting rabbits, 3 clinical units each. In all cases the rabbits went

into convulsions and showed a low blood sugar (Table IV), thus indicating very little if any alteration of insulin by the cerebrospinal fluid. This experiment eliminates the factor of insulin destruction by the cerebrospinal fluid. Probably the explanation is that owing to the large size of the insulin molecule it passes from the cerebrospinal fluid into the circulation at an exceedingly low rate. Our results are thus in accord with those of Dixon and fit in well with the studies of Abel and his collaborators who find that their crystalline insulin is of large molecular weight (6).

#### SUMMARY.

1. Insulin when injected subcutaneously causes a decrease in the blood and in the cerebrospinal fluid sugar and phosphates.
2. Insulin injected into the cerebellar cisterna causes only a slight, if any, decrease of sugar and phosphates in the blood, and, as a matter of fact, often results in an increase of these substances.
3. Insulin incubated with fresh rabbit cerebrospinal fluid does not lose its hypoglycemic properties.

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## RICKETS IN RATS.

### III. METABOLISM OF CALCIUM AND PHOSPHORUS OF RATS ON RESTRICTED FOOD INTAKES.

BY ALFRED T. SHOHL AND HELEN B. BENNETT.

(*From the Department of Pediatrics, Yale University, New Haven.*)

(Received for publication, May 23, 1927.)

The addition of phosphate to the diet of rats made ricketic on a high calcium-low phosphorus regimen, was followed by rapid lime salt deposition in the bones, and by an extremely high inorganic phosphorus and low calcium in the blood serum which resulted in spastic contractions of the limbs. The metabolism showed an increase in the absolute and relative amount of phosphorus retained (1). For the 1st week on the new food the rats consumed only one-third of the usual amount of food. Fasting for a short time has been shown to result in calcium deposition in the bones of ricketic animals (2). In animals on high calcium-low phosphorus diets, when food is withheld for 3 days, the phosphorus in the serum rises up to 13 to 16 mg. per 100 cc. and the calcium of the serum falls to 5.5 to 7.0 mg. per 100 cc. (Kramer and Howland (3), Cavin (4), and Harrison<sup>1</sup>). Data on limited food intakes are not available. The question arises, Are the observed effects due to the addition of phosphate or to the low food intakes?

The purpose of the present study was to determine the importance of restricted food intakes on the healing of rickets, and to consider further the metabolism of calcium and phosphorus. The animals were studied from the following points: (1) blood serum analyses, (2) histologic examination of the bones, (3) bone analyses, (4) metabolism.

#### *Conduct of Experiment.*

The study was conducted in June and July, 1926. Albino rats were removed from their mothers when 26 days old. Mothers and

<sup>1</sup> The data on fasting are taken from the unpublished thesis of Elizabeth Harrison, submitted in candidacy for the degree of Doctor of Medicine, Yale University, June, 1926.



young had access to the Sherman Diet B.<sup>2</sup> The young rats were continued on the same diet for 3 days. Animals of approximately the same weight were then selected. Four males and two females from two litters of ten and eleven rats born on the same day, were used for the experiment. On the 30th day these were given a mixture of one-half Sherman diet and one-half experimental diet. One rat was placed in each metabolism cage. The rats were fed the experimental diet for 2 days as a preliminary period and kept under observation to make sure that they were doing well and had become accustomed to the new cage. One rat was replaced because it spilled too much food.

The experimental diet was Steenbock's diet (No. 2965), modified by the addition of 10 per cent lard. This is a rickets-producing diet of the high calcium-low phosphorus type. The rats were continued on this diet for three periods of 6 days each. Then the diet was offered in daily amounts of only 2 gm. per rat, or one-third of the usual amount eaten. The rats were continued on this regimen for a fourth period lasting 6 days.

Whether an animal shows a steady and uniform gain or whether there are alternating periods of gains and losses can only be determined by frequent weighings. The animals were, therefore, weighed daily. Determination of daily food intakes is an advantage for the same reason, and food intakes for 6 day periods were necessary to compute balances for each period. The food was weighed daily and for each period.

The method of computing spillage was adequate for the requirements of the previous experiments. It is, however, more desirable to determine this factor by actual analysis, especially as the waste was greater in this experiment. The food remaining after the feces were picked out was, therefore, analyzed for calcium and phosphorus with that filtered from the urine.

Methods of analysis were the same as in the former experiment, except that the phosphorus was determined volumetrically by the method of Bang (5), as suggested by Greenwald (6). The yellow precipitate is dissolved in NaOH, the  $\text{NH}_3$  converted to hexamethylamine by formaldehyde, and the amount of phosphorus combined with base calculated from the amount of HCl required to titrate to neutrality.

<sup>2</sup> The references for these diets and the technique used are given in Paper I (Karelitz, S., and Shohl, A. T.), *J. Biol. Chem.*, 1927, lxxiii, 655.

In the analysis of the bones, femora of the rats were used whose actual metabolism had been studied. The fresh weight, dry weight, extracted weight, ash, and calcium determinations were made. The bones were first weighed fresh, and oven-dried at 105°C. to constant weight. They were broken in a mortar and reweighed before extraction. They were extracted for 36 hours with alcohol, for 12 hours with ether, weighed, and called "fat-free." The remainder was ashed in platinum in the electric muffle furnace below red heat to constant weight. The ash was dissolved in HCl, made to known volume and analyzed for calcium by the usual micro technique.

The experiment proceeded with no known errors. The animals were more peaceful than in the previous experiment. The food intakes were larger and the gains slightly better. When the food was limited to 2 gm. daily, the animals were very eager for the food and were more restless. During this period, spillage was negligible. The rats seemed weaker at the end of the experiment than when given diet *ad libitum* but were still in good condition. The food was insufficient to meet their needs and they lost weight in the last period.

### Results.

1. *Blood Serum Analyses.*—The blood serum findings show that after 21 days on the modified Steenbock diet and 3 days on the limited food intakes the calcium was 10.5 and the phosphorus 5.4 mg. per cent. After 6 days on limited intakes the calcium was 7.5 and the phosphorus 8.3 mg. per cent.

In the rickets control group the values are 10.5 to 12.0 mg. per cent for calcium and 3.5 to 4.5 mg. per cent for phosphorus. Hence the results obtained with limited food intakes lie between these values and normal values. This result differs markedly from that of fasting, and that of the addition of phosphate to the diet. With fasting the calcium drops to 7.0 to 7.3 mg. per cent and the phosphorus rises to 13.2 to 12.2 mg. per cent on the 3rd and 4th day. With phosphate added to the diet, the calcium drops to 7.0 to 8.4 mg. per cent and the phosphorus rises sharply to 16 to 12.7 mg. per cent on the 3rd and 7th days respectively. Thus in both cases the calcium values are far below normal and the phosphorus values far above normal. When food intakes are restricted the

serum of the rats shows normal calcium and subnormal phosphorus values.

The relationships can be most clearly seen in the graphs. In Fig. 1 the calcium of the serum is plotted for (1) added phosphate, (2) fasting, (3) rickets control, (4) limited diet. In Fig. 2 the phosphorus values are given. The curves indicate that although changes are induced by feeding one-third the usual amount of rickets-producing food, these values shift from those of rickets toward normal. They are quite different from the effect of added

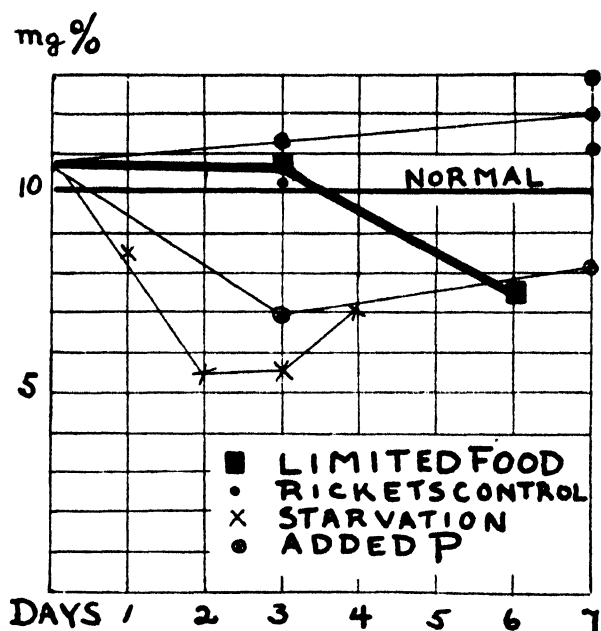


FIG. 1. The serum calcium of ricketic rats, showing effect of limited food intakes, fasting, and added phosphate.

phosphate and fasting, which give very low calcium and very high phosphorus values. Therefore, the conclusions of the previous experiment seem justified: the results were due to phosphate addition and not to limitation of intake.

2. *Histology*.—The bones after 3 and 6 days on limited intakes show marked rickets. There is a wide metaphysis and no evidence of healing. The sections in no way resemble those of the previous experiment when the food intakes were the same. Healing was then far advanced as a result of adding phosphate to the diet.

They also bear no resemblance to sections of rats in which calcification takes place as a result of fasting, the so called line test.

3. *Bone Analyses.*—Analytical data on the bones are given in Table I. The technique of Chick and Roscoe (7) was used. The actual weights per single femur are given, as well as the percentage values. The calcium values equal 13.4 per cent of the dry weight after 3 days on limited food and 15 per cent after 6 days compared to a normal at 65 days of 21.1 per cent. These values represent

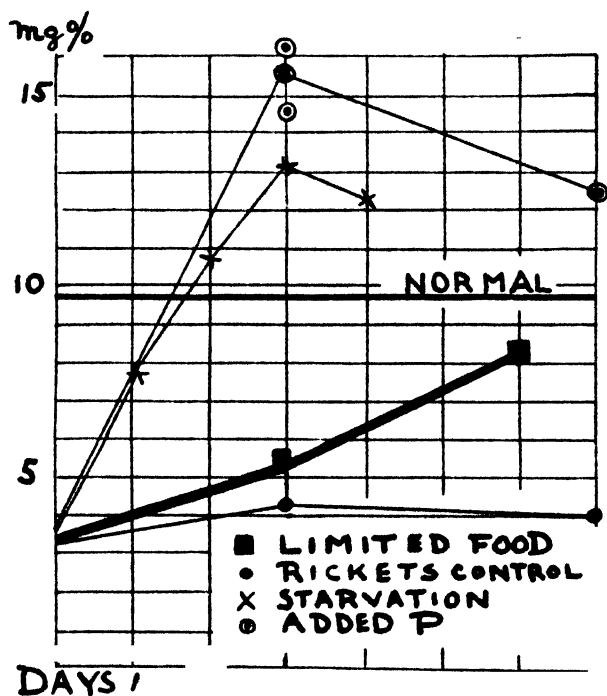


FIG. 2. The serum phosphate of ricketic rats, showing effect of limited food intakes, fasting, and added phosphate.

the same degree of calcium deposition as in the ricketic bones of the animals previously reported (1).

When the actual values are compared with Hammett's data (8), the bones of the rats after 3 and 6 days on limited food weigh respectively but 95 and 78 per cent of those of normal animals at the same age. The organic matter represents 130 and 90 per cent of the normal amount. Therefore, the ash is but 64 and 51 per cent and the calcium 65 and 53 per cent of the actual amounts which should be present at this age.

Steenbock (9) proposed the value of the ash of the fat-free bones as a quantitative measure of rickets. At 24 days the normal rat femur contains 45.0 per cent of ash. We have interpolated from his data, corroborated by Dutcher *et al.* (10), for the values at 54 and 57 days and calculate these respectively as 56.9 and 57.6 per cent. At the same ages the extracted femora of our experimental animals showed 38.5 and 41 per cent ash. This indicates faulty lime deposition. It corroborates the calcium analyses in the bones. Chick and Roscoe (7) have pointed out that ricketic control bones

TABLE I.  
*Analysis of Femur.*

Values in terms of one femur.

	Limited food intake.		Normal, 54 days.†	Normal, 57 days.†
	3 days.*	6 days.*		
	mg.	mg.	mg.	mg.
Wet.....	214	196	225	245
Dry.....	97	81	102	117
Fat-free.....	91	80		
Organic.....	62	47	47	52
Ash.....	35	33	55	65
Calcium.....	13	12	20	24

\* The animals were given modified Steenbock diet (No. 2965) for 21 days and then the same diet restricted in amount to 2 gm. per day. They were killed when 54 and 57 days old.

† These values are interpolated from Hammett's data for the average of males and females of the same age as the experimental animals.

contain more fat than do those of rats which had spinach as part of the diet and, therefore, calculate the percentage calcium on the fat-free basis. This gives higher values for the calcium calculated as per cent. No normal values for rats at this age are available as their rats on cod liver oil received only 1 to 3 drops per day. The bones were considered normal histologically, but contained only 19.4 per cent calcium on the dry basis or 20.9 per cent of the extracted bone. On the fat-free basis the bones of our rats showed 14.3 and 15.1 per cent calcium, thus indicating, because of the small amount of fat present, an even more severe degree of rickets than that estimated from the dry basis.

#### 4. *Metabolism of Calcium and Phosphorus. Weights of Animals.*

—The rats averaged 48.3 gm. at the outset of the experiment and 62.4 at the end of the 18 days on the modified Steenbock diet. This represents about one-half the optimal growth. At the end of the 6 day period on limited diet they weighed only 60.3 gm. Their average gain was 6.0 gm. for the first period, 4.8 gm. for the second period, 3.3 gm. for the third period. They lost 2.1 gm. for the fourth period.

The loss of weight during the period of greatly restricted intake is small compared to that suffered during fasting. Winters, Smith, and Mendel have found (11) that rats of this age must consume 125 per cent of the calories which the experimental animals received during this period to maintain just constant weight. Fasted animals seldom survive the 5th day and often die before that, but our animals were still in good physical condition on the 7th day.

*Food Intakes.*—The food consumed was slightly more than that reported in the first two studies which averaged 4.75 and 4.4 gm. per rat per day. For the first three periods in this study they averaged 7.1, 6.7, and 5.8 gm. gross daily and 6.3, 6.4, and 5.5 gm. net daily. The average net intake was 6.1 gm. The spillage is 7 per cent of the average gross and is 11.0, 4.5, and 5.0 per cent for each period. One rat spilled so much food that it was replaced after 3 days by another. The spillage decreased after the first period when they were becoming accustomed to the diet. During the fourth period the food was spilled in such small amounts and so little was left in the food cup that it can be said the food was eaten quantitatively. The character of the food was the same as that eaten in the three previous periods.

*Weight of Feces.*—The weight of the dried feces shows consistent amounts for each period and is slightly more than those previously reported, which averaged 3.6 and 3.8 gm. per rat per week. For the first three periods they equal 4.0, 4.4, and 4.0 gm. per rat per week. This is in agreement with the slightly better food intakes and gains in weight. During the fourth period the weight of the food was only one-third of the previous amount, but the feces equal one-half that of the previous periods. Thus the feces are more than proportional to the food intakes and represent some of the material furnished by the breakdown of body substance.

*Paths of Excretion.*—The data for the intakes and output of

calcium and phosphorus are given in Table II. They are calculated on the basis of one rat for 1 week. For the first three periods the amounts of calcium and phosphorus excreted in the urine and feces are quite similar to those found in our two previous experiments and corroborate them. They represent the effect of a diet high in calcium and low in phosphorus. Most of the calcium and phosphorus is in the feces; the urine was practically phosphorus-free. The ratio of Ca:P averages 5.4 in the feces. After restricted feeding the feces show a ratio of 3.0. This small alteration

TABLE II.

*Metabolism of Six Rats for Calcium and Phosphorus on Modified Steenbock Diet.*

Figures in terms of one rat per week.

Calcium.							Phosphorus.						
Period.	Intake.	Urine.	Stool.	Total output.	Balance.		Intake.	Urine.	Stool.	Total output.	Balance.		
<i>wks.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent of intake</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent of intake</i>	
1	512	73	280	353	+159	31	118	1	72	73	+45	38	
2	497	109	297	406	+91	18.3	115	1	82	83	+32	28	
3	427	112	258	370	+57	13.4	99	1	68	69	+30	30	
Average.	478	98	278	378	+102	21.3	111	1	74	75	+35	31.5	
4	150	44	89	133	+17	11.3	35	0	33	33	+2	5.7	

is due to the larger proportion of calcium in the urine and of phosphorus in the feces than occurred in the control periods.

*Calcium Balance.*—The balances of calcium are slightly larger in the first three periods than in the former experiments and correspond to the slightly better intakes and growth. In the first two experiments the balance during the period on the rickets-producing diet averaged 82 and 80 mg. Here the average value is 102 mg. In this experiment, however, as the food was measured for each period the balances can also be ascertained. These values are 159 mg. of calcium for the first period, 91 for the second, and 57

for the third, showing a diminishing positive balance as rickets progresses. Even on the limited intake the balance is still positive, 17 mg. per week. It represents only 11.3 per cent of the intake, however, and only 7 per cent of the normal retention.

*Phosphorus Balance.*—The balance of phosphorus is also positive and greater for the first three periods than in the previous experiments. It averages 35 mg. instead of 23 mg. per rat per week. The weekly phosphorus balances show, as do the calcium balances, a diminution as rickets progresses, and they also remain positive. During the fourth period phosphorus retention was at a minimum; only 2 mg., or 6 per cent, of the intake were retained. This represents only 2 per cent of the normal balance.

*Ratio of Retention.*—The ratio of the average calcium to average phosphorus retained for the first three periods is 2.9, with weekly values of 3.5, 2.85, and 1.9. The average value is similar to those found in the two previous studies. The weekly values indicate that the relative excess retention of calcium cannot proceed indefinitely. In the third period retention of calcium and phosphorus approaches a normal ratio. In the fourth period the retentions are so small that their ratio has little significance. An excess of calcium is still retained.

#### DISCUSSION.

Rats offered less rickets-producing food than they would willingly consume show rickets. Measured by blood serum determinations, bone histology, bone analyses, and calcium and phosphorus metabolism, their condition does not represent healing or a metabolism essentially different from that of rats having free access to the same diet. Eventually the animals would probably die on such limited intake. The effect of undernutrition from this diet is quite in contrast to that when phosphate was added to the diet and the animals self-limited their intakes. It must be concluded that the results previously reported were due not to fasting, but to the addition of phosphate.

#### CONCLUSIONS.

1. Rickets-producing food limited for 6 days to one-third of that normally consumed causes only slight changes in the ricketic rat. (1) The blood serum changes are toward normal. (2) The



bone histology is that of rickets without healing. (3) The bones show loss of calcium. (4) The positive balances of calcium and phosphorus are reduced to a minimum.

2. The changes reported (in Paper II (1)) are due not to fasting but to the addition of phosphate to the diet.

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## THE DETERMINATION OF CALCIUM IN WHOLE OXALATED BLOOD.

By CARMEN S. ROTHWELL.

*(From the Department of Pediatrics, Yale University, New Haven.)*

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To develop a method for the determination of calcium in whole oxalated blood which would be accurate and simple in execution was our purpose. The principle of the method was suggested by the fact that calcium oxalate is soluble in acid solution below a pH of 4.0 (1). If blood were treated with trichloroacetic acid a double function would be fulfilled; the proteins would be removed, and the calcium in whole oxalated blood would be brought into solution. An aliquot of such a filtrate was neutralized and used for the calcium determination. The rest of the procedure is essentially the same as in the direct method for serum first proposed by de Waard (2), the technique for which was worked out in this country by Kramer and Tisdall (3), and modified by Clark and Collip (4).

Whole blood can be prepared for the determination of calcium by three types of methods: (1) destruction of organic matter by ashing; (2) precipitation of the protein to furnish a protein-free filtrate; (3) direct determination in the presence of protein, but in the absence of cellular detritus.

Various modifications of ashing have been described (5-8) but a method which is equally accurate, and does not involve ashing, is to be preferred. Calcium determinations on the ash were obtained only as a check on filtrate values. The ashing method employed was a preliminary Neumann ashing with nitric and sulfuric acids, oxidation being hastened by the addition of 30 per cent  $\text{H}_2\text{O}_2$  (superoxol). The sulfuric acid was evaporated in a platinum dish, and the ash extracted with 0.1 N HCl.

Methods employing the protein-free filtrate have been proposed by Halverson and Bergeim (8), Lyman (9), and Kramer and

Tisdall (10). Halverson and Bergeim remove the protein from citrated blood by the use of picric acid, and precipitate calcium in an aliquot of the filtrate. The method of Halverson and Bergeim has been studied by Clark (11) and has been found to give positive errors, as high as 30 per cent. Lyman precipitates the proteins by pipetting whole blood directly into a weak solution of trichloroacetic acid. He determines calcium nephelometrically as calcium stearate. Denis and Minot (12) and Clark (11) report wide variation of calcium values by this method. Kramer and Tisdall hemolyze blood by pipetting into a weighed amount of water, weigh the blood added, precipitate the proteins with trichloroacetic acid, and determine calcium on the filtrate. Underhill and Dimick (13) and Underhill and Gross (14) have reported extensive studies on human blood and on dog blood using this method. Some of their values for whole blood calcium would be considered fairly high for serum alone. Clark (11), by deproteinization with trichloroacetic acid without the use of an anticoagulant, reports values from 10.5 to 30.5 per cent higher than the ash.

Clark (11) has developed a method for precipitating calcium in whole blood in the presence of protein, after hemolysis of the cells and removing the debris. He reports values higher by 1.5 to 9.0 per cent than those obtained by ashing in platinum.

#### *Method.*

Our final procedure is as follows: Blood is collected so as to contain about 0.3 per cent of potassium oxalate. Shake just before pipetting the sample, to insure uniform distribution of any precipitated calcium oxalate. Pipette 10 cc. into a 50 cc. volumetric flask, add about 15 cc. of distilled water and 12 cc. of 15 per cent trichloroacetic acid, rotating to insure complete precipitation of the proteins. Shake. Let stand 10 minutes. Add caprylic alcohol if the mixture foams, make to volume, and filter through a No. 40 Whatman filter paper. (Filtration is much facilitated and the amount of filtrate increased by centrifuging before filtration.) In 25 to 30 cc. centrifuge tubes, take two samples of 15 or 17.5 cc. of the filtrate (17.5 cc. which equals 3.64<sup>1</sup> cc. of blood, gives a

<sup>1</sup> The figure 3.64 instead of 3.50 is used, as the precipitate occupies 4 per cent of the total volume and the aliquot is corrected by that amount.  $3.50 \times 1.04 = 3.64$ .

satisfactory titration). Add 4 to 5 drops of concentrated ammonia, 2 drops of methyl red, and adjust to the neutral point, using first strong ammonia and approximately 50 per cent HCl, then weak ammonia and 0.1 N HCl for the final neutralization. Add 1 cc. of saturated ammonium oxalate and bring back to the proper pH with about 1 drop of 0.1 N HCl. Mix, and let stand  $\frac{1}{2}$  to 1 hour. Centrifuge and wash once, according to the technique of Clark and Collip. Titrate the precipitate with standardized 0.01 N  $\text{KMnO}_4$ .

*Calculation.*

Cc. of titration minus blank (0.03 to 0.05 cc.)  $\times$  0.2 mg. divided by cc. of blood in aliquot  $\times$  1.04 = mg. per 100 cc. of whole blood.

EXPERIMENTAL.

Many of our first filtrates from trichloroacetic acid precipitation ran high, checked against the ash. We were using a large excess of acid, but for convenience usually added the blood to the solution of trichloroacetic acid and water. Some of the filtrates thus prepared had a ground glass appearance and gave a faintly positive biuret reaction, which led us to question our method of protein precipitation. All filtrates decolorized a large amount of permanganate, as against no reduction by trichloroacetic acid itself. The work of Corley and Denis (15) suggested the use of permanganate for oxidizing this trace of protein. After preliminary oxidation with permanganate, values agreed within experimental limits with those obtained by ashing.

While studying the conditions best adapted for complete precipitation of proteins by our method, we included determinations by the methods of Halverson and Bergeim (8), Kramer and Tisdall (10), and Clark (11). The results are shown in Table I.

Of the methods at present available, that of Clark is the most accurate, and can also be executed with the greatest ease. The only difficulty which it presents is in washing the precipitate free of protein. If this is not done results will run high. If the blood mixture is precipitated and washed shortly after hemolysis, this difficulty is largely obviated. The high results obtained by the Kramer-Tisdall technique are due to the amount of acid used, which is insufficient to remove the proteins completely. Small amounts of incompletely precipitated protein remaining after

treatment with trichloroacetic acid are a source of error, although serum calcium can be determined in the presence of all the native proteins. The trichloroacetic acid should be added to the blood as recommended, for incomplete precipitation results if too little acid is added (16), or if the blood is added to the acid, instead of the acid to the blood. We have found that 0.8 volume of 15 per cent trichloroacetic acid approaches the lower limit so far as complete removal of proteins is concerned. We have, indeed, obtained correct values with this ratio of acid, but at other times results have been high. On the other hand, we have obtained correct values using an excess of acid up to 1.8 volumes of 15 per cent trichloroacetic acid to 1 of blood; with 2.5 volumes results

TABLE I.  
*Calcium in Whole Human Blood by Various Methods.*

Per 100 cc. of whole blood.						
Ash.	Halverson and Bergeim.	Error.	Clark.	Error.	Kramer and Tisdall.	Error.
mg.	mg.	per cent	mg.	per cent	mg.	per cent
5.92	7.72	+13.0			6.1	+3.0
5.10	8.62	+16.9			5.76	+10.3
6.30	6.40	+1.5				
5.50			5.94	+8.0	10.95	+19.9
			6.1	+10.0	11.5	+20.9
5.54			5.9	+6.0	6.9	+12.4
6.00			6.4	+6.5		

are low (Table II). With this method of deproteinization we have found, as have Hiller and Van Slyke (16), that the acidity of the filtrates is more acid than pH 1.0.

With a concentration of potassium oxalate not greater than 0.5 per cent accurate figures were obtained; with 0.7 per cent of oxalate, results were high (Table II). Correct results were obtained from blood containing 0.3 per cent of potassium oxalate after it had stood in the ice box 2 and 3 days, respectively. The blood was shaken by hand before pipetting the samples. A concentration of 0.2 per cent of potassium oxalate is sufficient to prevent coagulation, but a concentration of 0.3 per cent gives accurate results by our method and requires less care in handling. We have, therefore,

recommended this concentration, but it should not be higher. Sodium citrate gives low results, and cannot be employed in place

TABLE II.  
*Calcium in Human Blood.*

Values expressed per 100 cc.

Whole blood ash.	Serum hematocrit.	Serum calcium.	Whole blood calcium.	Error.	Remarks.
mg.	per cent	mg.	mg.	per cent	
5.74	60.9	9.3	5.66	-1.4	Serum, direct precipitation. Whole blood Ca calculated from serum and hematocrit, compared with the ash.
			5.62	-2	1.2 volumes acid, 0.3 per cent oxalate.
			5.56	-3	1.2 " " 0.5 " " "
			5.94	+3.5	1.2 " " 0.7 " " "
6.16	60.0	10.1	6.06	-1.6	Serum, direct precipitation. Whole blood Ca calculated from serum and hematocrit, compared with the ash.
			6.2	+0.6	0.3 per cent oxalate, 0.8 volume acid.
			6.14	-0.3	0.3 " " " 1.0 " "
			6.04	-0.3	0.3 " " " 1.2 " "
			7.15	-0.2	0.3 " " " 1.2 " " +
					1.0 mg. Ca.
			6.14	-0.3	0.3 per cent oxalate, 1.5 volume acid.
			6.14	-0.3	0.3 " " " 1.8 " "
			5.26	-14.6	0.3 " " " 2.5 " "
					Serum precipitated with:
			10.4*	6.25	+1.5 0.5 volume acid.
			10.2*	6.13	-0.5 0.8 " "
			10.4*	6.25	+1.5 1.0 " "
			10.1*	6.07	-1.0 1.2 " "
			10.2*	6.13	-0.5 1.8 " "

\* The serum filtrate values are uncorrected for the volume of the trichloroacetic acid precipitate. The whole blood figures given in the next column are calculated from the serum filtrate values and the hematocrit. The percentage error of the calculated whole blood is computed from the whole blood ash.

of potassium oxalate. Lithium citrate and lithium oxalate gave results that closely correspond to those obtained with potassium oxalate.

A correction must be applied to the values obtained because of the bulk of the precipitate. We have found that the actual volume of the fluid is 47.95 instead of 50; therefore, 4.1, plus or minus 0.1 per cent, must be subtracted from the value of the titration obtained; or, as we have preferred to consider it, 104 per cent of the calculated aliquot is taken. Thus 17.5 cc. of the filtrate represent 3.64 cc. instead of 3.5 cc. of the original blood. No correction has been evaluated for serum filtrates similarly obtained, but on the basis of the protein content a correction of 1 per cent is indicated.

In Table II we have used but two different samples of blood as illustrating the points in question. These determinations are no better than some hundred others, but as they were checked by ashing, by hematocrit and serum determinations, and by addition of calcium, we have chosen them as representative of the accuracy to be expected from the method. The method of ashing has been described above. The hematocrit values were obtained by centrifugation in a Daland hematocrit at high speed for 45 minutes. No correction for the shrinkage due to oxalate was applied. The assumption was made that the cells contained no calcium and the values for whole blood were calculated from hematocrit and serum calcium determinations. Calcium was added as  $\text{CaCl}_2$ , prepared from calcite.

With the proper acid concentration, oxalate content, and correction factor, the results for whole oxalated blood filtrates agree with the ash values, and the values obtained from serum and hematocrit determinations, as accurately as the method of washing and titration permits. Added calcium is recovered (Table II). The method gives correct values for calcium plus or minus 2 per cent.

#### SUMMARY.

A method is described for the determination of calcium on the trichloroacetic acid filtrate from oxalated blood.

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# ANIMAL CALORIMETRY.

THIRTY-FIFTH PAPER.

## ON THE MECHANISM OF PHLORHIZIN DIABETES.\*

By HARRY J. DEUEL, JR., H. ELLIS C. WILSON,† AND  
ADOLPH T. MILHORAT.

WITH THE TECHNICAL ASSISTANCE OF JAMES EVENDEN.

(From the Department of Physiology, Cornell University Medical College,  
New York City.)

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\* A preliminary report of most of these data was given at the meetings of the Federation of American Societies for Experimental Biology at Rochester, N. Y., in April, 1927.

† Fellow in Medicine of the Rockefeller Foundation.

## I. INTRODUCTION.

The explanation of the mechanism by which phlorhizin acts is a much debated question. There is little doubt that its primary action is exerted on the kidney, whereby the ability of the tubular cells to reabsorb the glucose normally present in glomerular urine is decreased. It is also assumed quite generally that phlorhizin directly inhibits the oxidation of carbohydrate.

The renal character of the action of phlorhizin was first proved by the classical experiments of Zuntz (1). Nash (2) found that the blood from the renal artery contained more sugar than that of the corresponding vein when phlorhizin was injected. This proved that the drug causes an increased permeability of the kidney for sugar rather than an increased production of glucose from non-protein sources, as postulated by Pavy, Brodie, and Siau (3) and by Levene (4). The results of Nash were later confirmed by Schenk (5).

Nash and Benedict (6) assume that the action of phlorhizin is not confined merely to the kidney but that it possibly forms a relatively stable union with the "carbohydrate receptors" of the cells. Consequently, this glucose can no longer combine with them and so result in oxidation. These investigators suggest that phlorhizin prevents the oxidation of glucose in a manner analogous to that by which carbon monoxide abolishes the ability of the hemoglobin to carry oxygen. Ringer (7), also Gaebler and Murlin (8), attribute the inability of the phlorhizinized animal to utilize glucose to the failure of the insulin supply. The former author believes that this drug acts specifically on the pancreas, preventing the production of insulin, rather than having a generalized effect on all the cells, while the latter workers suggest that phlorhizin neutralizes this hormone in the tissues rather than at the site of formation. Nash and Benedict (9) in a later communication disagreed with the hypothesis of Ringer because they were able to prepare potent extracts of insulin from the pancreas of animals which had received large amounts of phlorhizin over a number of days.

The hypothesis that phlorhizin causes an impairment in the cells to oxidize carbohydrate is based on several types of evidence. Of these the most striking is the frequent quantitative elimination of glucose in the urine when carbohydrates are given orally or introduced parenterally. Nash and Benedict (6) found an almost complete recovery of the ingested glucose when as much as 30 gm. were given in spite of a hyperglycemia which persisted in some cases as long as 6 hours. Ringer (10) obtained a 93 per cent return of glucose after the oral administration of 75 gm. of this substance in divided doses to a phlorhizinized dog. There are numerous experiments on record in which a practically complete elimination of ingested or injected glucose has been obtained, but smaller amounts were given (Stiles and Lusk (11), Csonka (12), Nash (13), Deuel and Chambers (14), and Wierzuchowski (15)). However, in many cases the recovery was only 90 per cent of that

introduced. When such results are obtained there is always a question as to whether the 10 per cent unaccounted for is to be traced to experimental difficulties inherent in the method or to possible oxidation. Such quantitative excretion of ingested carbohydrates is not always obtained, especially when large amounts are given. One of us (16) has obtained definite evidence with a completely phlorhizinized hog in which, when 200 gm. of glucose or 400 to 700 gm. of starch were given, the elimination of the ingested carbohydrate as glucose in the urine was far from complete, results which can be interpreted only to indicate that the phlorhizinized hog is able to oxidize and store considerable quantities of the ingested foodstuff.

Deuel and Chambers (14) reported that glucose rapidly restored the strength of phlorhizinized dogs weakened by hypoglycemia, although a quantitative elimination of the ingested carbohydrate was apparently obtained. Wierzuchowski (15) was the first to note that the administration of glucose to such animals was followed by the decrease or disappearance in the ketosis which ran parallel to the fall in the protein metabolism previously observed. He ascribed this phenomenon to the oxidation of a part of the ingested carbohydrate.

The second line of evidence which seems to indicate an impairment in the carbohydrate-oxidizing mechanism brought about by phlorhizin has been obtained from a study of the effect of glucose in producing an elevation of the R.Q. was noted by Lusk (17) during the 2nd hour after the administration of 75 gm. of glucose to a phlorhizinized dog nor for several hours after the ingestion of 10 gm. of glucose or fructose by such animals. Similar results were obtained by Michael Ringer with 40 gm. of this monosaccharide. More recently, Wierzuchowski (18) did find an increase in the level of the R.Q. of two phlorhizinized dogs for several hours after the administration of 20 gm. of glucose, a result which was especially marked when two 20 gm. doses were given 4 hours apart.

The results of Underhill (19) are also given to support the view that a phlorhizinized dog loses the power to oxidize carbohydrate. This investigator found a marked increase in the blood sugar value in these animals following the ligation of the renal vessels. The dogs lived only 12 hours after the operation, however, and the rise in this value might be attributed to the trauma of the operation or to a premortal hyperglycemia. The foregoing results are not in harmony with the earlier experiments of Minkowski (20) nor of those reported later in this paper.

In the experiments described in the present paper we have sought to throw more light on the mechanism of the action of phlorhizin by studying the effect of the injection of this substance on the R.Q. of dogs which had been fed with a high carbohydrate diet—a régime ordinarily giving basal R.Q.'s of approximately 1.00 to 0.90 in the morning 18 hours after the last food.

Experiments were also carried out on nephrectomized dogs which received phlorhizin daily and which were fasted, to ascertain whether the R.Q. was lowered to the diabetic level always found in the fasting phlorhizinized dogs with normal kidneys on the 3rd or 4th days after the first administration of this drug, or whether this value was only reduced to the fasting level of normal animals. The blood sugar and the rate of increase in the non-protein nitrogen of the blood have also been followed in these animals in order to determine whether phlorhizin affects these values any more than does nephrectomy alone. The results will be discussed under several sections.

## II. GENERAL PROCEDURE.

The effect of phlorhizin on the R.Q.'s of well fed dogs during fasting and directly after the administration of glucose was determined on female dogs. The animals which were nephrectomized were good sized males which had been fed with dog biscuit previous to the operation and which were fasted thereafter.

Nitrogen analyses of the urine were carried out by the usual macro Kjeldahl technique. Urine sugar was determined by the procedure of Bertrand, blood sugar by the Shaffer-Hartmann method, and non-protein nitrogen by the procedure outlined by Greenwald (21). Urine was collected by catheter. Blood for analysis was usually obtained from the saphenous vein or the median vein and in a few cases from the jugular vein. Care was taken to avoid exciting the animals in any way when the samples were withdrawn.

## III. EXPERIMENTAL PART.

### *1. Respiratory Quotients of Well Fed Dogs Immediately Following Injection of Phlorhizin.*

Two female dogs well trained for the calorimeter experiments were given at 5 p.m. daily a diet of beef heart (100 gm.), cracker meal (100 gm.), lard (20 gm.), and bone ash (10 gm.) for a period of 2 months previous to the beginning of the experiments recorded here. During the last 2 weeks sufficient sucrose was added to the diet to cause an increase to approximately unity in the basal

R.Q.'s, as determined during the morning and early afternoon hours. This was accomplished in one case, in which the animal weighed 10 kilos, by the daily addition of 50 gm. of this sugar to the ration, while in the second instance, in which the dog weighed 13 kilos, 75 gm. of additional carbohydrate were fed.

When a sufficient number of basal determinations had been obtained to ascertain the level of the R.Q. at this period after food,

TABLE I.

*Non-Protein Respiratory Quotient at Various Periods after Food.*

Hrs. after food.....	19	20	21	22	23	24	25	26	27	28	29
Hrs. after phlorhizin.....	2	3	4	5	6	7	8	9	10	11	12
Dog 27.											
Basal.*.....	0.99	0.99	0.90	0.88	0.85	0.86					
Basal + phlorhizin..	1.01	0.93	0.93	0.97	0.97	0.93	0.85	0.99	0.94	0.77	0.80
Dog 41.											
Basal.†.....	1.07	1.01	1.04	0.94							
Basal + phlorhizin.	1.10	1.01	0.92	0.83		0.82	0.76	0.76	0.79		
Hrs. after food.....	31	32	33	34	44	45	46	47	67	68	69
Hrs. after phlorhizin.....	14	15	16	17	27	28	29	30	50	51	52
Dog 27.											
Phlorhizin.....	0.83	0.77	0.74	0.74†	0.69						
Dog 41.											
Phlorhizin.....					0.73	0.76	0.73	0.73	0.69	0.72	0.71

\* Average of five experiments.

† Average of two experiments.

‡ In a later experiment, in which the injection of phlorhizin did not produce the usual glycosuria, we observed the output of nitrogen to be abnormally low, facts suggesting a renal damage due to the repeated administration of this drug in previous experiments. The R.Q.'s were found to be 0.95, 0.95, 0.83, 0.87, 0.86, and 0.81 on the 17th, 18th, 19th, 20th, 22nd, and 23rd hours, respectively.

phlorhizin was given just previous to placing the animal in the calorimeter with all the other conditions the same as in the basal experiments. In order that the effect of the phlorhizin might be at a maximum in as short a period as possible 1 gm. was dissolved in 20 cc. of a 1 per cent solution of sodium carbonate, warmed to 50°C., and injected subcutaneously. 1 gm. was also given in olive oil according to the usual Coolen method. That the effect of phlorhizin attained a maximum very soon after such a procedure

can be seen by reference to the results obtained from the urinary constituents of two other dogs in which the sugar excretion was followed hourly after the administration of this drug (see Tables V and VI).

The summarized results of the R.Q.'s found in these experiments are given in Table I.

There were no essential differences in the height of the R.Q.'s 19 to 24 hours after food, irrespective of whether or not phlorhizin was administered just previous to the beginning of the calorimeter experiment. The control experiment has not been carried out for a longer time than 24 hours after food. It is probable that the R.Q.'s of the phlorhizinized dogs in the period following fall at a faster rate than would those of normal fasting animals having equal glycogen stores at the beginning. In the former case the available carbohydrate is being exhausted not only by oxidation but also by excretion as glucose in the urine.

There can be little doubt that the height of the R.Q.'s obtained during the first 24 hours after the administration of phlorhizin demonstrates that this drug does not impair the ability of the cells to oxidize carbohydrate. Even after 24 hours there was sufficient of this foodstuff remaining in Dog 41 so that diabetic R.Q.'s were not obtained and the animal was oxidizing appreciable amounts of glucose. This animal oxidized on an average 0.53 gm. of glucose hourly during the period 26 to 29 hours after the first administration of phlorhizin.

The question which now arises is, How soon after its injection may it be assumed that phlorhizin exerts its maximum influence? It acts on the kidney cells to the greatest degree within an hour after it is first introduced (see Table V). If the drug is carried to this organ so quickly in amount sufficient to inhibit the normal renal functioning, why should not one suppose that its effect on all the cells would be as prompt?

From the data available on Dogs 27 and 41 it is easy to calculate the total quantity of glycogen present in the animal at the time of phlorhizin injection. Since the dog is presumably in a post-absorptive condition 18 hours after food, all the glucose oxidized or excreted subsequent to that time must be traced to glycogen or to protein. The total glucose formed from both of these sources is the sum of that which has been oxidized during the period, that

converted to fat, and that excreted in the urine. Two procedures are possible for the calculation of the portion which arises from protein. In the first place, one may assume that all the glucose originating from protein is oxidized previous to the time when the diabetic R.Q.'s are obtained. For this period one uses the normal factors for the calculation of the non-protein R.Q. Since one has assumed that the protein fraction of the glucose is completely oxidized, all of this carbohydrate present in the urine previous to the diabetic R.Q. must owe its origin to glycogen.

TABLE II.

*Metabolism of Dog 41 after Phlorhizin.*

Body weight, 13.1 kilos.

Hrs. after phlorhizin....	1	2	3	4	5	6	7	8	9	10	11-24
Non-protein R.Q...	1.10	1.10	1.01	0.92	0.83		0.82	0.76	0.76	0.79	
Glucose oxidized, gm.....	5.88	5.88	4.25	3.75	2.50	2.16	1.82	1.03	0.88	1.39	14.00
Hrs. after phlorhizin....	25	26	27	28	29	30-48	49	50	51	52	
Non-protein R.Q...		0.73	0.76	0.73	0.73			0.69	0.72	0.70	
Glucose oxidized, gm.....	1.00	0.36	0.89	0.42	0.44	5.70*	0.30*				

	gm.	per cent
Total glucose oxidized .....	52.65	= 47
" extra glucose excreted.....	54.49	= 49
Glucose to fat.....	4.18	= 4

Grand total..... 111.32

\* Estimated.

The glucose arising from protein subsequent to this time is determined by multiplying the urinary nitrogen by 3.65. Secondly, the same results may be arrived at by assuming that none of the glucose from the protein is oxidized after the administration of phlorhizin, under which condition one must use the diabetic values for the calculation of the non-protein R.Q. throughout. In this case the amount of glucose formed from protein is the product of the total urinary glucose and 3.65. It is probable that both of these assumptions are partially correct. Valid results of the glucose originating from protein are obtained by either of these



methods of computing. In the present case we have used the first procedure. If the glucose formed from protein is subtracted from the total carbohydrate accounted for, the balance must have arisen from glycogen. The summarized results on these animals are given in Tables II and III.

In the case of Dog 41, 52.65 gm. of glucose were oxidized before a diabetic R.Q. was obtained, 4.18 gm. were converted to fat, and 54.49 gm. were excreted in the urine (above that traceable to protein metabolism), which gave a grand total of 111.32 gm.

TABLE III.  
*Metabolism of Dog 27 after Phlorhizin.*

Body weight, 10.1 kilos.

Hrs. after phlorhizin..	1	2	3	4	5	6	7	8	9	10	11	12
Non-protein R.Q..	1.01	1.01	0.93	0.93	0.97	0.97	0.93	0.85	0.99	0.94	0.77	0.80
Glucose oxidized, gm.....	2.30	2.30	1.93	1.93	2.28	2.28	2.20	1.59	2.39	1.85	0.71	0.94
Hrs. after phlorhizin..	13	14	15	16	17	18-26	27					
Non-protein R.Q..		0.83	0.77	0.74	0.74		0.69					
Glucose oxidized, gm.....	1.08	1.22	0.66	0.44	0.44	3.15*	0.00					

Total glucose oxidized.....	gm.	per cent
" extra glucose excreted.....	29.69	= 47
	33.85	= 53

Grand total..... 63.54

\* Estimated.

of glucose arising from glycogen, or 0.85 per cent of the body weight (0.76 per cent as glycogen). With Dog 27, which weighed 10 kilos, the total glucose oxidized and excreted after the administration of phlorhizin amounted to 63.5 gm., or 0.63 per cent of the body weight (0.57 per cent as glycogen).

The values so obtained probably represent fairly accurately the store of glycogen in the well fed dog, although small amounts of this polysaccharide remain in the liver and particularly the muscles even after the animal becomes completely diabetic. The results which have been calculated for the total glycogen content

of the animals are somewhat higher than those which may be calculated from average analyses of Junkersdorf (22) for well nourished dogs fed with a diet containing materials in the same relations as Voit's dietary. The total glycogen content of the muscles, heart, kidneys, and liver of a 10 kilo dog was 43.6 gm., or 0.44 per cent of the body weight. Our slightly higher results may be caused by a greater food intake of the animals previous to the experimental period. Likewise, no allowance for the glycogen content of certain structures known to contain glycogen, such as the bones, is made in the calculation above. Our results are in close agreement with those of Schöndorff (23) who found that the total glycogen content in three well fed dogs varied from 0.58 to 0.76 per cent of the body weight. In four other experiments much larger amounts of glycogen were found by this investigator. The fact that the glycogen content determined by these two different experimental methods is of the same general order of magnitude, offers additional evidence that the extra glucose obtained in the early periods after the administration of phlorhizin can be traced to glycogen rather than to a production of carbohydrate from fat.

## *2. Effect of Glucose Ingestion on Respiratory Quotients of Fasting Phlorhizinized Dogs.*

In order to determine whether the ingestion of small amounts of glucose would elevate the R.Q.'s of dogs which had received phlorhizin and fasted for 4 or 5 days previously, ten experiments were carried out on three different animals with 16, 10, and 5 gm. of glucose. Preliminary determinations were made to demonstrate that the R.Q.'s had reached the diabetic level, but with the subsequent experiments the basal determinations were omitted. Table IV gives a summary of the experimental results. Additional data giving the urinary glucose and nitrogen values necessary for the calculation of the extra sugar in these experiments are recorded in Protocol 1.

In almost every case there is some rise in the R.Q. in the hours immediately following the administration of glucose. In the experiments with Dog 41 in which 16 gm. of glucose were given on 3 successive days there is a considerable rise in the R.Q. during the 2nd and 3rd hours of the 1st day and the 2nd hour of the 3rd

TABLE IV.  
*Influence of Ingestion of Various Amounts of Glucose upon Respiratory Quotients in Phlorhizinized Dogs. (Diabetic Factors Used (Lusk (17)).*

Dog No.	Experi- ment No.	Body weight.	Non-protein R.Q.						D: N ratios.		Glucose.		
			Before glucose.			After glucose.			Before.	After.	Extra recovered in urine.	Oxidized.	Total accounted for.
			2 hrs.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.					

Experiments with 16 gm. glucose.														
41	18, 19	kg.	0.707	0.707	0.790	0.793	0.699		3.94	3.82	12.67	3.28	20.5	15.95
41	20	12.45			0.756	0.761	0.730	0.699	3.82	3.66	14.38	2.57	16.1	16.95
41	21	12.10			0.804	0.759	0.759	0.688	3.66	3.38	14.61	3.67	22.9	18.28
49	1, 2	15.10	0.715	0.715	0.785	0.751	0.751		3.79	3.46	15.28	4.07	25.4	19.35
49*	3, 4	13.90			0.733	0.756	0.710	0.707†	3.46	3.52	15.85	1.89	11.8	17.74
									Average 14.56			3.10	19.3	17.65

Experiments with 10 gm. glucose.														
27	57, 58	9.70	0.713	0.709	0.739	0.753	0.733	0.739	4.53	4.06	9.27	1.65	16.5	10.92
27*	59	9.55			0.735	0.722	0.765	0.713	4.06	†		1.30	13.0	
41*	24	11.65			0.725	0.729	0.732	0.743	3.99	4.51	8.31	1.49	14.9	9.80
41*	25	11.10			0.740	0.724	0.724	0.732	3.51	†	7.76‡	1.32	13.2	9.08

Experiment with 5 gm. glucose.														
41*	22, 23	11.90	0.720	0.726	0.719	0.709			4.25	4.20		0	0	

\* Phlorhizin in  $\text{Na}_2\text{CO}_3$  given before glucose.

† Average of 6th and 7th hours after glucose.

‡ Dog very weak; experiment not continued.

§ Urine collection for 8 hours only.

day but much less of a change on the 2nd day. This suggests that there is some variability with the same animal under identical conditions in utilizing glucose. With this animal the R.Q. always returned to the basal level the 4th and 5th hours after glucose was given.

That this rise in R.Q. is not to be attributed to the oxidation of  $\beta$ -hydroxybutyric acid seems proved from the experimental results of Wierzuchowski (18). He found that when the acidosis was abolished by a preliminary administration of glucose an increased R.Q. followed the second dose of sugar although no change in the  $\text{CO}_2$ -combining power of the blood then resulted. According to the theoretical calculations of this investigator the oxidation of  $\beta$ -hydroxybutyric acid may set free sufficient alkali to bring about a decrease rather than an increase in the R.Q.

When 10 gm. of sugar were given to Dog 41 and Dog 27 there was a slight rise in the R.Q. which was not so great nor so uniform as that obtained with 16 gm. There seems to be a definite effect, however. When 5 gm. of glucose were given to Dog 41 no increase in the R.Q. was detectable during the period in which the experiment was made.

In some of the experiments phlorhizin was administered in sodium carbonate just previous to the introduction of the sugar in order that it might exert its maximum effect on the kidney soon after the injection and in this way cause a more rapid elimination of glucose. In one experiment (Dog 49) this method seemed to decrease the extent of the oxidation, while in a second case scarcely any effect could be traced to its introduction. However, in no instance did it entirely prevent the oxidation of carbohydrate.

It is obvious from these results that, whereas an average recovery of 14.6 gm. of glucose as extra sugar in the urine after the administration of 16 gm. *per os* constituted a recovery of 90 per cent, yet if one adds to this the amount of glucose oxidized one obtains a sum which represents an average of 17.6 gm., or 10 per cent more than was given by mouth. On one occasion when 16 gm. of glucose were given 19.3 gm. was the amount calculated to have been removed from the body either through the kidney or by oxidation. This sum is 20 per cent more than that theoretically obtainable.

The discrepancy between the amount of carbohydrate fed and

that accounted for is too great to be ascribed to the experimental errors in the methods employed. It is conceivable that the increased concentration of glucose in the tissues or blood following the administration of carbohydrate to the phlorhizinized dog permits the oxidation of muscle glycogen already present. Should this be the case, the increased R.Q.'s observed after the ingestion of 16 gm. of glucose may be attributed wholly or in part to the oxidation of muscle glycogen.<sup>1</sup>

Lusk (24) has shown that, after establishing the D:N ratio of 3.65 in a completely phlorhizinized dog weighing 18 kilos, the action of shivering will remove 14.5 gm. of extra urinary glucose presumably derived from glycogen, for Prausnitz (25) has reported that a dog weighing 22 kilos, after fasting 12 days and after excreting 287 gm. of glucose brought about by the repeated injections of phlorhizin, still contained 25 gm. of glycogen in its body.

It is quite possible that the lower R.Q.'s found in our laboratory (17) after giving 10 gm. of glucose and fructose were due to the preliminary sweeping out of glycogen through exposure of the phlorhizinized dog to shivering, a process which was previously systematically employed in the experimental work of this laboratory. Using this method of preparing the dog, Csonka (12) was able to recover 16.15 gm. of extra glucose in the urine in the 7 hour period after the oral administration of 16 gm. of this carbohydrate.

Further experimental work is in progress to determine whether the increased R.Q.'s which we have observed in the present experiments are to be traced to a stimulation of the oxidation of the muscle glycogen or to the oxidation of the ingested glucose. A report of these experiments will appear later.

### *3. Rate of Glucose and Nitrogen Elimination Following Phlorhizin Injection.*

In order to determine with what speed the maximum effect of phlorhizin is exerted on the kidney and how soon the increase in the nitrogen metabolism becomes evident, the following experiments were carried out on two female dogs which were fed with the same diet as Dog 41 and in which the phlorhizin was injected at

<sup>1</sup> This suggestion was offered by Prof. S. R. Benedict in a personal communication.

TABLE V.

*Glucose and Nitrogen Excretion in Hourly Periods before and after Injection of Phlorhizin in Dog 49.*

Body weight, 16.9 kilos.

Hrs. before or after phlorhizin.	Urine volume.	Glucose.	Nitrogen.	D:N ratio.	Extra glucose.	Blood sugar.
	cc.	gm. per hr.	gm. per hr.		gm. per hr.	mg.
4-6*	12	†	0.175			0.103
3*	15	†	0.176			0.103
2*	18	†	0.187			0.108
1*	10	†	0.165			

1 gm. phlorhizin injected in 20 cc. 1 per cent  $\text{Na}_2\text{CO}_3$  followed by 1 gm. in 10 cc. oil.

1	49	2.650	0.194	13.25	1.942	
2	34	2.977	0.167	17.83	2.367	0.103
3	49	3.209	0.183	17.54	2.541	0.105
4	37	2.948	0.176	16.75	2.306	0.098
5	36	3.000	0.183	16.39	2.332	
6	36	2.942	0.178	16.53	2.292	0.100
7	32	2.971	0.182	16.32	2.307	
8	31	2.816	0.177	15.91	2.170	0.102
9	27	2.792	0.185	15.09	2.117	
10	27	2.982	0.194	15.37	2.274	0.105
11	29	2.788	0.195	14.30	2.076	
12	26	2.794	0.208	13.43	2.035	0.101
13	24	2.649	0.211	12.53	1.879	
14	27	2.627	0.241	10.90	1.747	
15	25	2.321	0.284	8.17	1.284	0.086
16	21	1.983	0.340	5.83	0.742	
17-18	24	1.961	0.408	4.80	0.472	0.077
19-20	23	2.072	0.475	4.36	0.338	0.100
21-24	20	2.240	0.484	4.63	0.473	0.082
25-43		2.364	0.577	4.10	0.258	0.093
44-67		2.398	0.595	4.03	0.226	0.059
68-95		1.885	0.505	3.73	0.042	0.055
96-99‡		4.695‡	0.405‡	11.60‡	3.177‡	0.173‡§

Average glucose per hr. (2-12) = 2.929 gm.

" " " " (13-95) = 2.191 "

\* Hours before phlorhizin.

† Negative qualitative test (Benedict's).

‡ After 16 gm. of glucose *per os* the 96th hour.

§ Maximum blood sugar 97th hour.

approximately the same interval of time after food. Blood sugar determinations were made at frequent intervals and are reported in detail in Section 4.

TABLE VI.

*Glucose and Nitrogen Excretion in Hourly Periods before and after Injection of Phlorhizin in Dog 169.*

Body weight, 9.8 kilos.

Hrs. before or after phlorhizin.	Urine volume.	Glucose.	Nitrogen.	D:N ratio.	Extra glucose.	Blood sugar.
	cc.	gm. per hr.	gm. per hr.		gm. per hr.	mg.
3*	5	†	0.107			
2*	5	†	0.090			0.102
1*	6	†	0.088			0.101

1 gm. phlorhizin in 20 cc. 1 per cent  $\text{Na}_2\text{CO}_3$  and 1 gm. in oil, both subcutaneously.

1	12	1.20	0.078	15.33	0.92	0.118
2	18	1.52	0.089	17.04	1.22	0.100
3	17	1.74	0.096	18.08	1.39	0.118
4	19	1.76	0.107	16.70	1.37	0.114
5	21	1.75	0.109	16.61	1.35	0.118
6	18	1.68	0.114	14.74	1.26	0.097
7	15	1.73	0.121	14.30	1.29	0.094
8	22	1.80	0.137	13.14	1.30	0.106
9	20	1.86	0.150	12.40	1.31	0.108
10	27	1.84	0.159	11.57	1.26	0.107
11	26	1.90	0.162	11.73	1.31	0.107
12	21	1.86	0.156	11.92	1.29	
13-24	50	1.33	0.197	6.72	0.61	0.071†
25-48	38	1.37	0.288	4.75	0.32	
49-72		1.35	0.356	3.80	0.05	
73-75§		4.67§	0.336§	13.92§	3.47§	0.254§

Average glucose per hr. (2-12) = 1.77 gm.

" " " " (13-72) = 1.35 "

\* Hours before phlorhizin.

† Negative qualitative test (Benedict's).

‡ Amount for the 24th hour after phlorhizin.

§ After 16 gm. glucose *per os* the 73rd hour.

|| Maximum blood sugar 74th hour.

Table V gives the essential data which were obtained on Dog 49, while Table VI reports a similar experiment on Dog 169.

In the experiment recorded in Table V the action of the phlor-

hizin on the kidney occurs so promptly that during the 1st hour after its injection the glucose excreted is approximately 90 per cent of the average for the following 9 hours during which time the sugar excretion is at the maximum. With Dog 169 (Table VI) the glucose eliminated during the 1st hour is only 68 per cent of that excreted in the following 11 hours. In both instances the maximum effect was obtained during the 3rd hour, although the values for the 2nd hour are only slightly lower.

The rate of glucose excretion is very constant throughout the first 12 hours, during which period the blood sugar level is normal. The constancy of the elimination of this urinary constituent suggests that the action of the phlorhizin is such that the kidneys are excreting at a rate that is maximal for the blood sugar value at the time. With a hyperglycemia following the ingestion of glucose a greater amount of this monosaccharide may be eliminated in a given time than when the blood sugar is at a normal level.

It is of interest to compare the value of the urinary nitrogen with that of the blood sugar. Is the blood sugar the factor controlling the increase in the protein metabolism or is it due to some action of the phlorhizin? The first evidence of the increase in the height of the protein metabolism becomes evident 12 hours after the phlorhizin administration and while the blood sugar is still at the same level as in the prephlorhizin period. The urinary nitrogen has increased 50 per cent 15 hours after the phlorhizin, while the blood sugar has fallen only from 0.101 to 0.086. The latter value is a perfectly normal one and is not associated with an increased protein catabolism in normal fasting dogs. Similar results were obtained with the second animal. The blood sugar value fluctuates somewhat but does not reach the hypoglycemic level until 44 hours after the first injection of phlorhizin. Therefore it would seem that the blood sugar cannot alone be the controlling factor in determining the degree of protein metabolism in phlorhizinized animals. That such is not a normal cause is suggested by the slight effect which insulin hypoglycemia produces on the protein metabolism.

There is an immediate diuresis in the case of Dog 49 coincident with the excretion of the glucose in the urine. The highest values for the D:N ratio were obtained the 2nd or 3rd hour after the injection of phlorhizin and fell gradually until the glycogen stores



were largely exhausted at the end of 24 hours. In one case (Dog 49) the D:N ratio had then reached the fasting level. In depancreatized dogs Chambers and Coryllos (26) found that the first increase in nitrogen excretion which could be attributed to the pancreatectomy occurred 12 hours after the operation and was concurrent with the onset of glycosuria. The highest D:N ratios found in these animals were obtained 15 to 18 hours after the removal of the pancreas, which indicated an early depletion of the glycogen supply.

#### *4. Effect of Phlorhizin on the Blood Sugar Level of Normal and Nephrectomized Dogs during Fasting and after Glucose.*

The experiments recorded below were carried out to determine whether phlorhizin decreases the ability of the tissues to hold their glycogen or whether the great depletion of this substance in the tissues of animals so treated is to be traced to the continual breaking down of the glycogen reserve in their attempt to maintain the blood sugar at a normal level. Although the latter explanation seems the more probable and is the more widely accepted, there is some evidence to indicate that glycogen can less readily be formed in the dog made diabetic with phlorhizin than in the normal animal. Csonka (27) has found that when glucose was administered to a phlorhizinized dog in which the renal vessels had been ligated there was an increase in the blood sugar value from 0.058 to a maximum of 0.290. On the other hand, the ingestion of the same amount by a normal dog under identical conditions only resulted in a rise in the blood sugar from 0.075 to 0.090. These results would indicate that the liver and tissues of the phlorhizinized animal are less able to synthesize and store glycogen than those of the normal animal. Such a condition might be due to the action of phlorhizin *per se* or to the acidosis incident to the diabetic metabolism resulting from its injection.

If phlorhizin acts on the liver to cause a flushing out of the glycogen in the same manner in which adrenalin does, an immediate increase in blood sugar should follow shortly after its injection. On the other hand, if glycogenolysis occurs only for the purpose of preventing the onset of a hypoglycemia, the introduction of this drug should not produce an increase in the blood sugar level. However, the rise in blood sugar might be prevented by the rapid

excretion of glucose in the urine, which would mask any glycogenolytic action of the phlorhizin. Such a condition can be excluded by the previous ablation of the kidneys. A primary action of this drug on the glycogenolytic mechanism must then be evidenced by an increased blood sugar level.

The blood sugar values of two well fed normal dogs and of two nephrectomized animals have been followed at frequent intervals

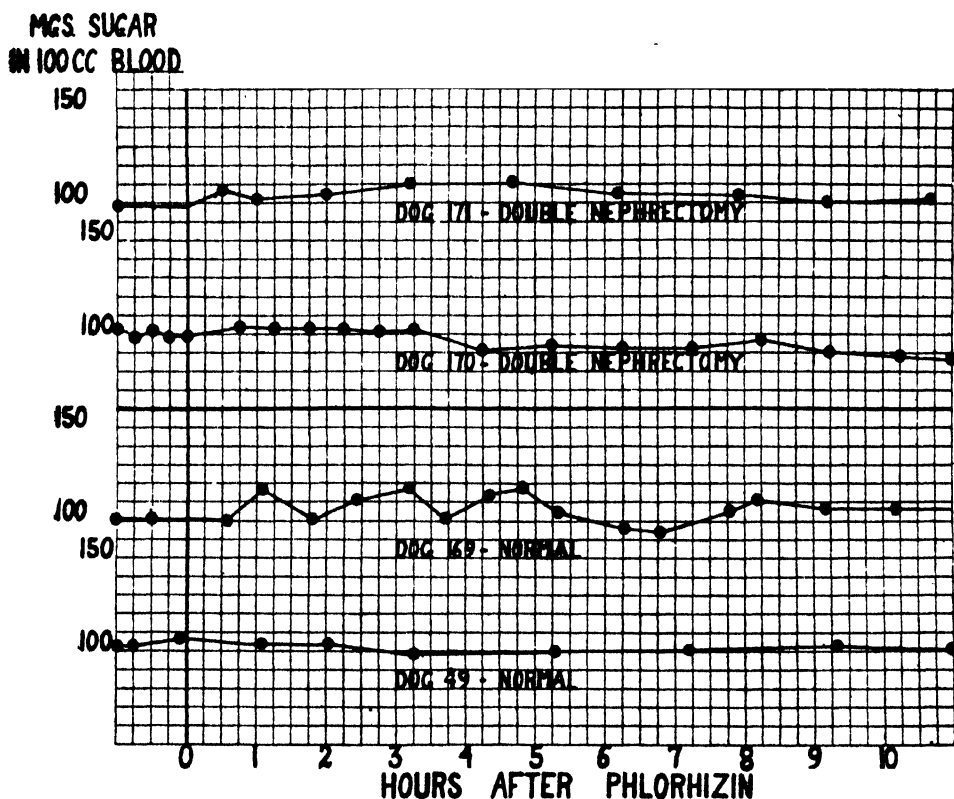


CHART 1. The effect of phlorhizin on the blood sugar of normal and nephrectomized dogs.

over periods of 12 hours after the injection of phlorhizin. The results of these experiments are given in Chart 1.

No increase in the blood sugar level occurred after the injection of phlorhizin either in the normal dogs or in the nephrectomized ones for a period as long as 12 hours. In the former case a gradual drop in the blood sugar followed as the excess of the carbohydrate was drained out in the urine. This resulted in a hypoglycemia

on the 3rd day. There was no marked change in the height of this value in the blood of nephrectomized dogs until just prior to death. These experiments indicate that phlorhizin *per se* does not cause any breakdown of the glycogen reserves.

The effect of the administration of 16 gm. of glucose on the blood sugar levels of the two normal animals was followed on the 5th day of phlorhizin. The results are plotted in Chart 2.

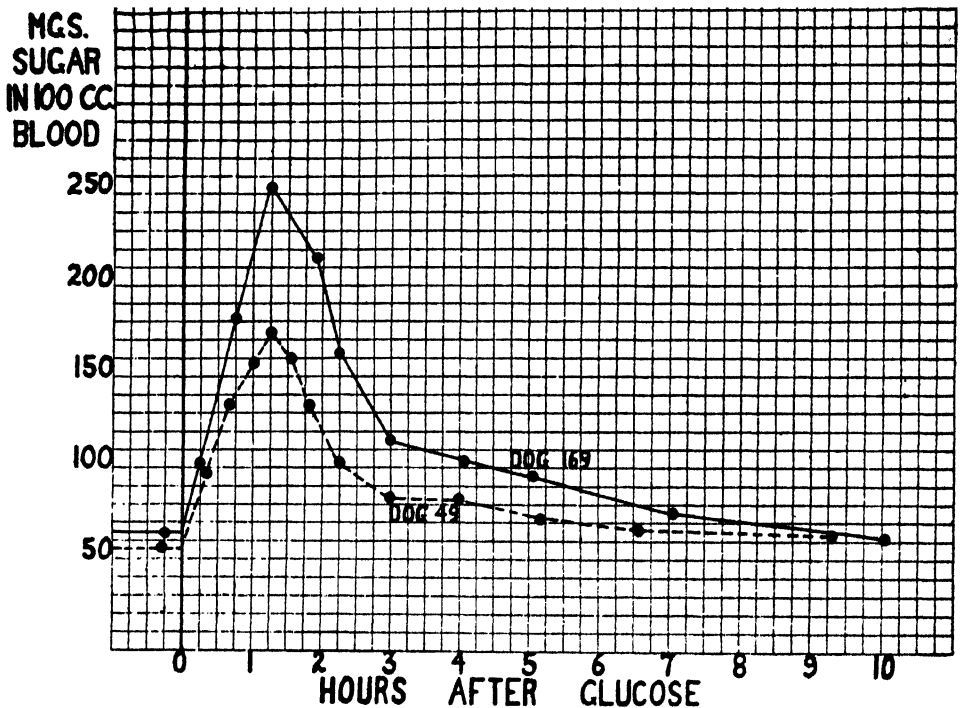


CHART 2. The blood sugar of dogs after the oral administration of 16 gm. of glucose on the 5th day of phlorhizin.

The ingestion of 16 gm. of glucose resulted in a rapid and very pronounced rise in the blood sugar level of Dog 169, reaching the maximum value of 0.254 per cent  $1\frac{1}{2}$  hours thereafter and remaining well above the normal level for 3 hours. The value did not return to the original hypoglycemic figure for nearly 10 hours. With Dog 49 the effect was much less pronounced than with Dog 169, but the hyperglycemic values were obtained for the same length of time and did not return to the original level any more rapidly.

These experiments confirm the observations of Guion and

Benedict (28) and of Nash and Benedict (6) in showing that a hyperglycemia is as readily brought about in a phlorhizinized animal with the ingestion of glucose as in the case of a normal dog.

*5. Effect of Phlorhizin on Respiratory Quotients of Fasting Nephrectomized Dogs.*

If the action of phlorhizin is solely a renal one, then its injection in nephrectomized animals should not cause any alteration in the carbohydrate metabolism. On the other hand, if this drug acts

TABLE VII.

*Effect of Phlorhizin on Respiratory Quotient of Nephrectomized and Normal Fasting Dogs.*

Dog No.	Condition of animal.	Length of fast preceding phlorhizin.	Body weight.	Approximate time after phlorhizin.			
				24 hrs.	48 hrs.	72 hrs.	96 hrs.
		hrs.	kg.				
47	Nephrectomized.	71	15.6		0.768 (3)	0.746 (3)	0.735 (2)
48	"	41	18.2	0.731 (2)	0.729 (2)	0.720 (2)	0.722 (2)
41	Normal, well fed previously.	18	13.1	0.715 (4)	0.691 (3)		
27	Normal, well fed previously.	18	10.1	0.715 (3)	0.699 (2)		

The figures in parentheses indicate number of experimental hours from which average R.Q.'s, as given, were obtained.

on the cells to prevent them from oxidizing carbohydrate, diabetic R.Q.'s should be obtained in these animals after several daily injections.

Therefore, we have carried out the following experiments to ascertain to what level the R.Q. fell after the extirpation of the kidneys of dogs when phlorhizin was administered daily to them when fasting. The kidneys<sup>2</sup> were removed through a median abdominal incision about 4 inches long. The renal vessels were ligated and the renal end of the ureter sewed to the surrounding

<sup>2</sup> The authors are much indebted to Prof. J. E. Sweet for his skill in performing the operations on our animals.

tissue. The animals lived from 4 to 6 days after the operation. The results of the operation were always confirmed at autopsy.

The R.Q.'s were followed on two of these animals daily after the administration of phlorhizin until the animals died. The summary of these experiments is recorded in Table VII.

The values for the basal R.Q.'s<sup>3</sup> in both nephrectomized animals which had received phlorhizin were usually above 0.73 and never lower than 0.72 even after the drug had been administered for 5 days. These figures are much higher than the diabetic R.Q. of

TABLE VIII.

*Comparison of Respiratory Quotients of Nephrectomized Dogs Receiving Phlorhizin with a Normal Dog Fasting the Same Length of Time.*

Dog No.	Nature of experiment.	Days after food.						Authority.
		3	4	5	6	7	8	
47	Nephrectomy; phlorhizin.			0.768	0.746	0.735		This paper.
48	Nephrectomy; phlorhizin.	0.731	0.729	0.720	0.722			" "
15	Normal.	0.710*	0.738	0.731*		0.738	0.712	Anderson and Lusk (29).

\*Work during this period.

0.69 obtained on the 2nd or 3rd day after the first injection of phlorhizin in dogs with normal kidneys. It will be noted that the phlorhizin was first introduced in our nephrectomized animals at a much longer interval after the last food than in the two normal

<sup>3</sup> The R.Q.'s reported on the nephrectomized animals are the observed ones rather than the non-protein values given in the earlier experiments in this paper, since there is no accurate way to calculate the extent of the protein metabolism in the animal without kidneys. With the diabetic animals the non-protein R.Q. is higher than the observed values in proportion to the height of the nitrogen metabolism. With normal animals the non-protein value on quotients under 0.80 is lower, and on quotients above 0.80 is higher than that of the total R.Q. Therefore, it is possible to have a non-protein R.Q. of a diabetic animal of approximately the same height as one of a fasting normal animal, although in the first case no carbohydrate is undergoing oxidation, while in the second instance it is taking place.

dogs which had been fed heavily 18 hours before. Therefore these animals must have utilized as much carbohydrate as dogs fasted for the same length of time and with normal kidneys would have oxidized, a corollary which can only be interpreted to indicate that phlorhizin did not inhibit the normal carbohydrate metabolism under these conditions. Table VIII gives the comparison of the R.Q.'s of our nephrectomized dogs and a fasting dog of Anderson and Lusk (29).

TABLE IX.

*Respiratory Quotients after Ingestion of Glucose in Nephrectomized Dogs 47 and 48 Receiving Phlorhizin, as Compared with a Normal Fasting Animal.*

Dog No.	Experiment No.	Day of fast.	Time after first phlorhizin.	Glucose given.	R. Q. after glucose.					Remarks.
					1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	
			hrs.	gm.						
47	3	6	72	16		0.869	0.810			Dog vomited.
48	3	4	56	20		0.748*	0.748*	0.763*	0.763*	
48	5	5	80	30		0.799	0.777			Dog vomited 1 hr., 10 min. after glucose.
27		12		30	0.817	0.831	0.757	0.710		Normal dog (Deuel, Waddell, Mandel (30)).

\*Average of 2 successive hours.

The height of the R.Q.'s in our nephrectomized dogs which received phlorhizin are quite in harmony with the values obtained by Anderson and Lusk for the fasting normal dog.

In order to determine whether small amounts of carbohydrate might elevate the R.Q. to a greater extent than it would in normal phlorhizinized dogs in which large amounts of the glucose are rapidly lost in the urine, experiments were carried out with these two animals after the ingestion of 16, 20, and 30 gm. of glucose. The results are summarized in Table IX.

The administration of 16 gm. of glucose to Dog 47 caused such a marked increase in the R.Q. as to leave no doubt that a large

proportion of carbohydrate was oxidized. Moreover, in both experiments with Dog 48 there was a definite increase in the oxidation of carbohydrates. In the second case an R.Q. as high as 0.80 was obtained after 30 gm. of the monosaccharide were given. The failure of a greater rise in the R.Q. in Experiment 3 with the second animal is probably to be attributed to the loss of a considerable amount of the glucose by regurgitation. Vomiting was constantly present in the nephrectomized animals, which made it difficult to obtain complete absorption of the carbohydrate.

The results of these experiments confirm the view that the nephrectomized animals receiving phlorhizin have not lost the power to oxidize carbohydrate. They behave as do normal fasting dogs. The results are quite in harmony with those obtained by Deuel, Waddell, and Mandel (30) with a normal fasting dog which received 2 to 3 times the amount of glucose per kilo of body weight that our animals did in the present experiments.

#### *6. Effect of Phlorhizin on the Non-Protein Nitrogen of the Blood and the Blood Sugar after Nephrectomy.*

One factor which is characteristic of the action of phlorhizin on dogs is the marked rise that is caused in the protein metabolism. Lusk (31) has shown that the protein breakdown may be increased to 450 per cent above the fasting level after the administration of this drug. As a usual condition the increase in the nitrogen excretion is scarcely apparent on the 1st day (see Table VI) and may not reach the maximum value until the 3rd day after its first injection, during which time the rise has been a progressive one.

This augmentation in the nitrogen metabolism following phlorhizin must be inherently related to the deficiency in the oxidation of the carbohydrate. On the other hand, the process may be reversed when carbohydrate is administered to a completely phlorhizinized animal. When small amounts of glucose are given, under such conditions, there is a gradual fall in nitrogen output which we feel is to be traced to a slight oxidation of this foodstuff, even though a practically quantitative recovery of the ingested sugar may apparently be obtained. This effect of carbohydrate is strikingly illustrated in the phlorhizinized hog (16), in which the urinary nitrogen was lowered from a value of 10 gm. daily to

approximately 2 gm. *per diem* for 2 days following the ingestion of 400 gm. of starch.

Therefore, if phlorhizin does not prevent the nephrectomized animal from utilizing glucose, it should occasion no alteration in the protein metabolism. The rate of increase in the non-protein nitrogen of the blood, which is the best index of this value in the animal without kidneys, should simulate that of control dogs which have had the kidneys extirpated but which have received no phlor-

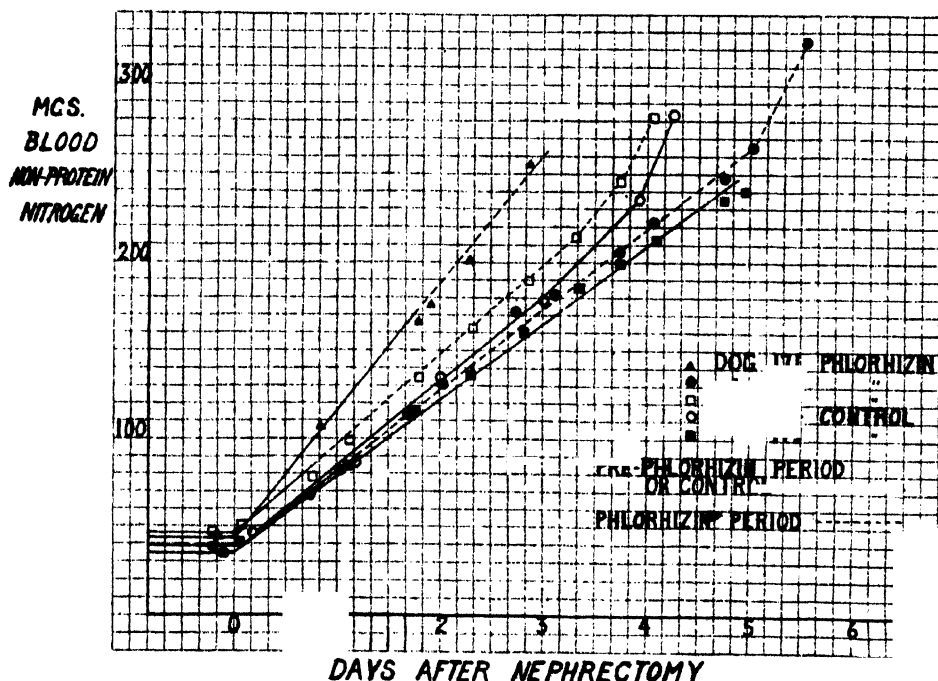


CHART 3. The non-protein nitrogen of the blood in nephrectomized dogs with and without phlorhizin.

hizin. If the nitrogen metabolism remained constant rather than progressively increasing after the injection of phlorhizin, the curve of these values should be a straight line rather than a parabola. The figures for the non-protein nitrogen of the blood of three nephrectomized dogs receiving phlorhizin and of two control animals are plotted in Chart 3.

There was no difference in the rate of increase in the non-protein nitrogen of the blood of nephrectomized dogs, irrespective of



whether or not the animals received phlorhizin. Likewise, the curve for these values in the dogs which received this drug showed no tendency to be parabolic but was practically straight. There was a slightly faster rate of increase in the non-protein nitrogen

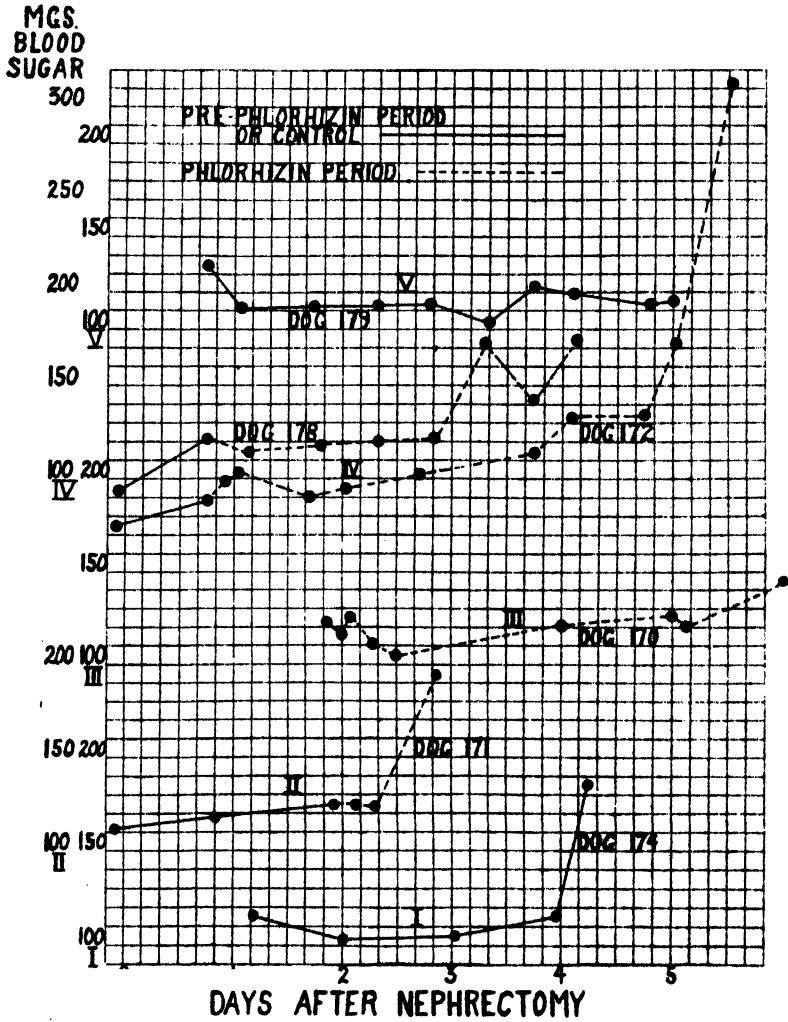


CHART 4. The blood sugar of nephrectomized dogs with and without phlorhizin.

just before death, which is probably to be traced to a concentration of the blood brought about by a negative water balance caused by the persistent vomiting. In our experience the blood taken just prior to death is very viscid and cannot be obtained from the

peripheral veins, which facts suggest such a concentration. These results, therefore, can be interpreted only to indicate that phlorhizin did not prevent the oxidation of carbohydrate.

If the injection of phlorhizin prevents the cells from such oxidation an increased concentration of glucose should occur progressively in the blood and tissues of nephrectomized dogs in which the avenue for excretion of the glucose by the kidney is removed. This increase should parallel that of the non-protein nitrogen of the blood, since in fasting protein forms the only source of glucose. In order to obtain further evidence as to whether phlorhizin prevents the oxidation of carbohydrate we have followed the blood

TABLE X.

*Glycogen Content of Nephrectomized Dogs with and without Phlorhizin.*

Dog No.	Condition of animal.	Length of fast.	Weight of animal.	Glycogen.				
				In liver.		In muscles.		Sum of both.
				per cent	gm.	per cent	gm.	
178	Nephrectomized; phlorhizinized 4 days.	121	19.2	0.234	1.30	0.266	20.40	21.70
179	Nephrectomized.	141	13.6	0.025	0.11	0.124	6.75	6.86

sugar in six nephrectomized dogs, four of which received this drug, and two of which served as controls. The data are plotted in Chart 4.

There was no progressive rise in the blood sugar level during periods as long as 4 days after the first injection of phlorhizin. In three cases out of four, with the animals receiving the phlorhizin and with one of the control dogs, there was a premortal increase in the blood sugar value which is probably to be traced to a concentration of the blood. The level of the earlier samples showed a slight hyperglycemia which was of the same degree for the phlorhizinized dogs as for the controls. The elevation in the blood sugar value just prior to death with animals in which the renal vessels have been ligated has been noted previously by Morita (32).

The only possibility which might be suggested to explain the

failure in the elevation of the blood sugar if glucose were not oxidized in these animals after the administration of phlorhizin is that the excess was prevented from accumulating in the blood by its prompt conversion to glycogen. That such was not the case with our animals was shown by the small amount of glycogen actually found in the liver and muscle tissue of one of the nephrectomized dogs which received phlorhizin. The summarized results are given in Table X.

TABLE XI.  
*Alcohol Checks.*

Date.	Experiment No.	Duration.	R. Q.	Calories.	
				Indirect.	Direct.
1926					
		hrs.			
Dec. 17.....	234	3	0.666	25.10	25.43
“ 27.....	235	2	0.666	25.76	25.30
1927					
Jan. 17.....	236	3	0.664	28.72	
Feb. 7.....	237	4	0.655	16.22	15.61
“ 8.....	238	3	0.660	23.49	25.05
“ 24.....	239	3	0.672	22.22	24.73
“ 28.....	240	5	0.650	17.44	17.39
Mar. 1.....	241	4	0.663	33.61	33.84
“ 19.....	242	3	0.662	35.09	33.76
Average.....			0.661	24.28	24.53

The total glycogen content of Dog 178 at the time of death amounted to approximately 22 gm., of which 1 gm. was in the liver and the balance was in the muscle tissue—if we assume the latter to comprise 40 per cent of the body weight. From the calculations of the glucose excretion by Dog 49 for 4 days after phlorhizin<sup>4</sup>

<sup>4</sup> The total glycogen content which would have been found had no glucose been oxidized was calculated as follows:

	<i>gm.</i>
Glucose excreted by Dog 49 (16.95 kilos) 4 days, 2 hrs.....	220.6
“      calculated for Dog 178 (19.2 kilos).....	249.9
“      oxidized by Dog 41 (13.1 kilos).....	52.7
“      “      calculated for Dog 178.....	77.2
Total glucose for Dog 178. ....	327.1
Glucose calculated as glycogen.....	294.4

and from similar computations of the glucose oxidized by Dog 41 (see Table II), the total glycogen content of Dog 178 should have been roughly about 294 gm. at the time of death if none of the glucose formed after the administration of phlorhizin had been oxidized. This is far in excess of that actually found in the animal. Therefore these results prove that carbohydrate must have been oxidized in the nephrectomized dogs receiving phlorhizin, since the blood sugar failed to rise and the analyses of the liver and tissues showed that the glucose formed could not have been stored away as glycogen to any great extent.

The presence of acetone is a constant occurrence in fasting phlorhizinized dogs with the renal function intact. We have been able to detect this odor in the breath of the animals in every case on the 3rd day of phlorhizin. However, in not a single instance with the nephrectomized dogs was there any suggestion of an acetone breath, even 5 days after the first administration of the phlorhizin.

#### *7. Alcohol Checks.*

The validity of the results obtained with the respiration calorimeter during this period is attested to by the alcohol checks carried out on the apparatus at frequent intervals. A summary of these results is given in Table XI.

#### IV. DISCUSSION OF RESULTS.

The experiments reported in the present paper were conducted to throw further light on the problem as to whether phlorhizin glycosuria is entirely a renal condition or whether it also involves an inability of the cells to metabolize carbohydrate. If the action of phlorhizin is only on the kidney, then the non-combustion of carbohydrate usually associated therewith, both during fasting and after the ingestion of small amounts of this foodstuff, are merely secondary phenomena which are dependent on the first condition. The ketosis and the high protein oxidation belong in the same category.

All the results which we have obtained indicate that the renal mechanism is the only one primarily involved in the action of phlorhizin. The height of the R.Q.'s in dogs fed with the standard diet plus sucrose was found to be normally between 1.00 and 0.90

18 hours after food and was unaffected by phlorhizin for a long period thereafter despite the fact that the maximum action of the drug on the kidney occurred 2 hours after its administration. In other words, when sufficient carbohydrate is present phlorhizin does not prevent its oxidation. Likewise, in completely phlorhizinized animals the rise in the R.Q.'s after 16 or 10 gm. of glucose suggested that some oxidation of the monosaccharide may have taken place.

When the kidneys are removed phlorhizin should exert no effect on the carbohydrate-oxidizing mechanism if it has no action on the carbohydrate receptors of the cells. Nephrectomized animals were found to be perfectly normal both in the height of the fasting R.Q.'s which were obtained and in their response to small amounts of glucose administered orally. These animals behave as do normal dogs fasted for the same length of time. Moreover, other factors indicated that the phlorhizinized nephrectomized animals were not diabetic. The nitrogen metabolism was exactly that of the controls which received no phlorhizin. Acetone was not produced as in the ordinary phlorhizinized animal. Blood sugar values, which must necessarily have risen if the animal did not oxidize carbohydrate and had no means of excreting it, were constant. This indicates that the nephrectomized and phlorhizinized animal must have oxidized glucose normally. The conclusion is obvious that if nephrectomized animals behave normally with respect to the carbohydrate metabolism when phlorhizin is administered, the same would be true of normal animals if sufficient carbohydrate were given to replenish the glucose lost in the urine.

The strongest evidence which is offered for the hypothesis which postulates that the cells are unable to oxidize glucose is the frequent quantitative recovery of ingested glucose in the urine. If we accept the fact that glucose is ordinarily entirely excreted instead of being only 90 per cent eliminated, is it not possible that such may occur even though the carbohydrate receptors of the cells were not affected? Even when glucose is given in moderate amounts to a subject which has fasted, the degree of oxidation is slight because it is quickly removed from immediate catabolism by storage as glycogen in the liver and tissues. In the phlorhizinized animal there are two avenues of escape from oxidation

for ingested glucose. In the first place a large proportion is rapidly removed from the field of possible oxidation by excretion as extra sugar in the urine. The second mechanism which reduces the oxidation to some degree is the immediate and temporary storage of the glucose as glycogen, which is later excreted when the blood sugar falls, as the experiments of Nash (13) indicate. Why should we not suppose, in a phlorhizinized animal in which the cells are far more depleted in glycogen than after a corresponding period of fasting, that glycogen is very readily formed from the excess of glucose so introduced?

That glycogen formation takes place temporarily after the administration of carbohydrate to a phlorhizinized animal seems certain. The best evidence of this is the finding of increased amounts of this polysaccharide in the liver and muscles after feeding carbohydrate to animals so treated (13). It is also indicated in our experiments in which 16 gm. of glucose were given to phlorhizinized dogs. Extra sugar is excreted in such experiments for as long as 12 hours after the ingestion of the monosaccharide, although absorption from the gut must be complete within 4 hours according to Fisher and Wishart (33). Such a condition is also strikingly demonstrated in the case of the phlorhizinized hog (16) in which extra sugar was being eliminated in the urine the 3rd day after the administration of 200 gm. of glucose, although its absorption must have required 10 hours at a maximum.

The results of the experiments reported here indicate that some oxidation of glucose may occur when 16 or 10 gm. are given to a completely phlorhizinized dog but not when 5 gm. are fed. When the former amounts are administered the proportion which is so oxidized is small in comparison with that ingested. Even though a slight oxidation of carbohydrate may occur under such conditions it is not of a sufficient magnitude to alter materially the recovery of extra glucose actually found from that theoretically to be expected if none was oxidized. This justifies the usual practice of assuming that the highest recovery in a series of experiments represents most nearly the true result. However, too fine distinctions in the interpretation of slight variations in the recovery of extra glucose should not be drawn when this method is employed.

The evidence obtained by us both with normal and with nephrectomized dogs indicates that the action of phlorhizin is entirely

a renal one. Carbohydrates are not oxidized during fasting or after the ingestion of small amounts of glucose, not because there is any intrinsic impairment in the mechanism for their oxidation, but on account of the low concentration of glucose in the blood and tissues. Glycogenolysis occurs in the attempt to compensate for the glucose lost in the urine after the injection of phlorhizin. This drug possesses no direct glycogenolytic action. This source of glucose likewise proves to be inadequate and the blood sugar falls to a distinctly hypoglycemic value. When this occurs carbohydrate ceases to be oxidized and diabetic R.Q.'s are observed. The cause for the non-oxidation of glucose under such conditions is problematical. One may assume that its concentration in the blood is too low to stimulate the pancreas to produce the insulin necessary for such oxidation. Another possibility is that a colloidal combination of glucose in the blood may protect this remaining amount of blood sugar from oxidation. However, the latter assumption is greatly weakened by the demonstration that normal blood contains readily diffusible glucose (34). This hypothesis also fails to explain how the blood sugar may be reduced to a negligible amount by excessive doses of insulin if some of the glucose is protected in a non-oxidizable combination. An incomplete oxidation of the fat results from the failure of the carbohydrate oxidation with the resulting ketosis. Were phlorhizin without its primary effect on the kidney, none of the foregoing phenomena, which follow its injection in normal animals, would occur. That such a hypothesis is cogent seems proved by our evidence that phlorhizinized nephrectomized dogs which are fasted behave exactly as do normal fasting animals with respect to the carbohydrate metabolism.

#### V. SUMMARY.

1. When phlorhizin (1 gm. in olive oil and 1 gm. dissolved in 1 per cent  $\text{Na}_2\text{CO}_3$ ) was injected subcutaneously into well fed dogs 18 hours after the last food no alterations were noted in the height of the R.Q.'s, which were found to be between 1.00 and 0.90 as in control experiments. The maximum effect of the phlorhizin on the kidney occurred the 2nd hour after its administration. The R.Q. had not reached the diabetic level 30 hours thereafter, but was usually at that value after 48 hours. The diabetic condition was

maintained by the injection of 1 gm. of phlorhizin in olive oil every 24 hours.

2. When glucose in doses of 16 and 10 gm. was given to completely phlorhizinized dogs which exhibited diabetic R.Q.'s, an increase in the height of the R.Q. was usually obtained. Generally this returned to the diabetic level after 4 hours. The glucose oxidized when 16 gm. were administered amounted to between 1.89 and 4.07 gm. and averaged 3.1 gm. The average excreted as extra urinary sugar in the experiments in which the larger amount was ingested was 14.56 gm., making a total of 17.65 gm. for the carbohydrate oxidized and excreted, or 10 per cent greater than that theoretically possible. A completely satisfactory explanation of this cannot as yet be given. No oxidation of carbohydrate was noted when 5 gm. were given.

3. The basal R.Q.'s of two fasting nephrectomized dogs were determined daily for 4 or 5 days after the administration of phlorhizin, which was first injected 18 to 24 hours after the operation. These values usually amounted to 0.73 or more and never reached a level lower than 0.72, in contrast to the diabetic R.Q. of 0.69. This indicates that the introduction of phlorhizin does not prevent as large an amount of carbohydrate from being oxidized as would have been utilized by a normal fasting dog without the injection of this drug.

4. After the administration of glucose in comparatively small amounts to these animals a rise in the R.Q.'s of varying degrees followed. In one instance after the oral introduction of 16 gm. of glucose it reached the values of 0.87 and 0.81 in 2 successive hours.

5. The blood sugar in the nephrectomized dogs remained practically constant at slightly hyperglycemic values after the injection of phlorhizin until just prior to death, when it increased somewhat, presumably due to blood concentration. A similar hyperglycemia and the same premortal rise were noted in the control animals which were nephrectomized but which received no phlorhizin. The glycogen in the liver and muscles of one of these animals was estimated to be about 22 gm. This amount is far too small to indicate that glucose is not oxidized in the nephrectomized phlorhizinized animals.

6. The rate of increase in the non-protein nitrogen of the blood was the same in the control animals and the phlorhizinized ones



with ablated kidneys, indicating that there was a constant protein metabolism in both cases. Had phlorhizin prevented the oxidation of carbohydrate, one would expect a much more rapid rate of increase in the non-protein nitrogen of the animal so treated than in the control.

7. No acetone could be noted in the breath of any of the nephrectomized dogs receiving phlorhizin even after 5 days, while such can always be detected on the 3rd day after normal dogs have been so treated.

8. These data are all in harmony in supporting the hypothesis that the action of phlorhizin is entirely renal; that there is no impairment in the ability of the tissues to oxidize glucose when present in normal quantities; and that the non-oxidation of this foodstuff usually observed in phlorhizinized animals is not due to the inability to utilize it but to the deficiency in the supply. A more complete treatment of this hypothesis is given in the discussion.

The authors wish to thank Professor Graham Lusk for his suggestions during the course of the experiments.

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## PROTOCOL 1.

*Effect of Administration of Glucose to Completely Phlorhizinized Dogs.*

Days after phlorhizin.	Length of period.	Glucose.	Nitrogen.	D:N ratio.	Extra glucose.	Prevailing D:N ratio.	Remarks.
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Dog. 41. Weight, 13.0 kilos.							
	hrs.	gm.	gm.		gm.		
4	19	23.18	5.89	3.94			
4	12	26.07	3.50	7.45	12.67	3.88	16 gm. glucose <i>per os</i> . Calorimeter Experiment 19.
5	12	12.81	3.35	3.82			
5	12	26.16	3.15	8.30	14.38	3.74	16 gm. glucose <i>per os</i> . Calorimeter Experiment 20.
6	12	13.17	3.60	3.66			
6	13	24.81	2.90	8.56	14.61	3.52	16 gm. glucose <i>per os</i> . Calorimeter Experiment 21.
7	12	14.26	4.22	3.38			
5	11	14.84	3.72	3.99			
5	10	20.37	3.22	6.33	8.29	3.75	10 gm. glucose <i>per os</i> . Calorimeter Experiment 24.
6	14	17.74	3.04	3.51			

Dog 49. Weight, 16.95 kilos.							
	hrs.	gm.	gm.		gm.		
4	28	50.31	13.48	3.73			
5	4	19.58	1.69	11.60	13.23	3.76	16 gm. glucose <i>per os</i> . Blood sugar in Chart 2.
6	16	26.91	7.10	3.79			
6	11	33.32	4.97	6.70	15.28	3.63	16 gm. glucose <i>per os</i> . Calorimeter Experiment 2.
7	12	19.01	5.49	3.46			
7	8	26.49	3.05	8.69	15.85	3.49	16 gm. glucose <i>per os</i> . Calorimeter Experiment 3.
8	16	25.78	7.33	3.52			

Dog 169. Weight, 9.8 kilos.							
	hrs.	gm.	gm.		gm.		
4	24	31.61	8.31	3.80			
5	6	20.95	2.09	10.02	13.26	3.68	16 gm. glucose <i>per os</i> . Blood sugar in Chart 2.
5	18	19.55	5.51	3.55			

## CONCERNING CHLORIDE DETERMINATIONS BY THE MODIFIED VOLHARD TITRATION.

By JOHN C. WHITEHORN.

(From the Biochemical Laboratory of the McLean Hospital, Waverley,  
Massachusetts.)

(Received for publication, June 3, 1927.)

For the benefit of those who use the writer's modification of the Volhard titration for determining blood or plasma chlorides,<sup>1</sup> the following brief communication is submitted in response to the recent adverse criticism by Short and Gellis.<sup>2</sup>

As these workers have noticed, the chloride method proposed by the writer "is not in all respects ideal." This does not distinguish it from many useful and reliable analytical procedures, which require care to avoid error. Had they merely recorded their personal preference for the iodometric titration which has some advantage in delicacy, no comment would be called for, but they state that: "The end-point in the Whitehorn method is unsatisfactory, as it yields results which usually are too low." Their published data are too scanty to furnish any clue as to the source of error they encountered. They suppose, without submitting experimental evidence, that their negative error was "due to the stirring and consequent increase in surface area of the silver chloride precipitate."

I have felt obliged to investigate this rather surprising supposition, since, if true, it would invalidate not only the method they criticize but also the methods developed by Van Slyke<sup>3</sup> and by Rehberg,<sup>4</sup> who, after confirming the writer's experience, have adopted this titration procedure.

If stirring produces the effect supposed, a titration with very

<sup>1</sup> Whitehorn, J. C., *J. Biol. Chem.*, 1920-21, xlv, 449.

<sup>2</sup> Short, J. J., and Gellis, A. D., *J. Biol. Chem.*, 1927, lxxiii, 219.

<sup>3</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1923-24, lviii, 523.

<sup>4</sup> Rehberg, P. B., *Biochem. J.*, 1926, xx, 483.

vigorous stirring ought to require more sulfocyanide than a duplicate with very gentle stirring. For this comparison I combined a considerable volume of blood filtrates, deproteinized by the Folin-Wu technique, and ran a number of titrations, alternating between (a) the gentlest stirring which would remove the red color after each drop of sulfocyanide, and (b) an extremely vigorous stirring after each drop, with an additional 5 minutes vigorous stirring midway in the titration.

The results, expressed as mg. of NaCl per 100 cc. of blood, were:

Mild stirring.	Vigorous stirring.
510	509
513	510
515	513
513	508
	508
	510
	513
	516
Average.....	511
512	

These results provide adequate refutation of Short and Gellis' unsupported assumption that stirring produces a negative error.

It is possible these workers may have been led by their unjustified fear of stirring, to neglect it so much as to produce an error by occlusion. One can thus manage to produce an appreciable negative error in the chloride estimation, as is indicated in the following results, obtained on the same pooled filtrate as was used in the mild stirring *versus* vigorous stirring experiments:

Minimum stirring.
495
497
495
497
502
497
Average.....
497

In this series almost all the sulfocyanide solution required was run into the titration mixture at one time, without any stirring at all until very near the end-point, and then stirred very gently. The writer assumes that the negative error which it is possible to produce in this way is due to the occlusion of sulfocyanide reagent within the precipitate of silver sulfocyanide. To avoid this one

need merely stir, however vigorously he likes, during the titration, as shown above.

I do not know whether this was the source of Short and Gellis' errors, but their fear of stirring suggests that possibility.

If there are others who have felt uncertain about the end-point in the sulfocyanide titration, and have not taken the time to check against a pure sodium chloride or potassium chloride solution, it is here suggested that the silver nitrate and sulfocyanide solutions, after adjustment to equivalence, be checked for reassurance against 10 cc. of 0.01 N HCl solution. "Any well trained routine laboratory technician"<sup>2</sup> may reasonably be supposed to have at hand a standard hydrochloric acid solution, accurate to at most 1 per cent, which can be appropriately diluted in a few minutes to 0.01 N. If desired, a drop or more of 10 per cent sodium tungstate solution may be added in a parallel determination to demonstrate its harmlessness.

I take this opportunity for three remarks which may be of value to those doing chloride determinations.

1. Brown nitric acid should not be used in the sulfocyanide titration as the lower oxides of nitrogen interfere with the end-point. This difficulty was so well recognized<sup>3</sup> that the writer considered it unnecessary to remark about it in the original paper. If the standard silver solution is prepared from metallic silver, it should be boiled to remove nitrous fumes.

2. In accordance with the excellent and growing practice of stating the results of blood analyses, particularly inorganic, in terms of molar concentration, I now use and recommend the use of standard silver and sulfocyanide solutions of 0.03 M concentration instead of M/35.46. The calculation then becomes

$$\text{mM concentration of chlorides} = 30(5.00 - \text{titer}).$$

3. For chloride determinations on spinal fluid which contains very little protein, a few minutes digestion with silver nitrate and nitric acid, as in Van Slyke's method,<sup>3</sup> disposes of the protein so simply that it is preferable to the tungstic acid precipitation. For the whole blood or plasma the tungstic acid deproteinization offers a saving in time and trouble, especially when one is also determining other substances included in the Folin-Wu system.

<sup>2</sup> Sutton, F., *Systematic handbook of volumetric analysis*, Philadelphia, 10th edition, 1911, 146. Dr. Elmer Sevringhaus also informs me that one of his pupils independently discovered this source of error.



# THE CARBON DIOXIDE EQUILIBRIUM IN ALVEOLAR AIR AND ARTERIAL BLOOD.

## II. RESTING SUBJECTS.\*

By D. B. DILL,† L. M. HURXTHAL, C. VAN CAULAERT, A. FÖLLING,  
AND A. V. BOCK.

*(From the Medical Laboratories of the Massachusetts General Hospital,  
Boston.)*

(Received for publication, June 6, 1927.)

In the first paper of this series (1) it was shown in resting subjects, by analyses of simultaneous samples of air and blood, that Haldane-Priestley samples collected at the end of normal expiration measure approximately the carbon dioxide pressure of arterial blood. The experiments cited in that paper justify the use of the method as a criterion for judging the reliability of other methods of collecting alveolar air. The occasional need for a method which requires no cooperation on the part of the subject has led us to compare the results obtained by the above method with those which various automatic methods yield.

We first studied the automatic modification of the Müller valve described by Henderson and Haggard (2) and used by them in their ethyl iodide method of measuring rate of blood flow. In our first experiments this was set up with the interposed ethyl iodide sampling tube of 250 cc. capacity and used as they have directed. We shall refer to it elsewhere as the HH valve and to the Haldane-Priestley method as the HP method.

We found, as they have reported, that the percentage of carbon dioxide in the collecting system becomes constant after 10 minutes use. None of our experiments ran for less than 10 minutes; the duration of most of them was 12 minutes or longer.

\* The expenses of this research were defrayed in part by the Tutorial Fund of Harvard University.

† National Research Fellow in Chemistry.



TABLE I.  
*Comparison of Haldane-Priestley with Henderson-Haggard Automatic Method of Collecting Alveolar Air.*  
 All subjects resting and reclining.

Subject.	Weight. kg.	Remarks.	No. of complete experiments.	Average ventilation. liters per min.	Respiratory rate per min.	Average tidal air. cc.	R. Q.	HP. Average pCO <sub>2</sub> . mm. Hg	HH value with interposed 250 cc. tube. Average pCO <sub>2</sub> . mm. Hg	pCO <sub>2</sub> HP - pCO <sub>2</sub> HH.		
										Average. mm. Hg	Maximum. mm. Hg	Minimum. mm. Hg
A.V.B.	68	Basal.	5	5.30	5	1070	0.84	39.2	33.9	+5.3	+6.9	+3.4
D.B.D.	72	"	9	4.80	10	480	0.84	42.2	37.9	+4.3	+5.6	+3.1
J.S.L.	62	"	6	4.70	11	430	0.83	40.7	36.2	+4.5	+5.9	+3.9
L.M.H.	80	"	6	7.15	8	630	0.82	42.6	38.5	+4.1	+6.4	+2.5
A.V.B.	68	Not basal.	4	7.00	12	580		41.3	34.8	+6.5	+8.4	+3.1
D.B.D.	72	"	10	6.00	12	500		42.2	37.9	+4.3	+5.7	+3.4
J.S.L.	62	"	1	5.20	14	370		40.6	34.5	+6.1		
L.M.H.	80	"	2	6.70	9	740		42.9	38.4	+4.5	+5.3	+3.6
H.J.R.	73	Polyserositis.	1	6.25	12	520	0.87	33.7	30.9	+2.8		
M.F.	57	Exophthalmic goiter.	3	6.30	21	300	0.75	41.1	32.3	+8.8	+9.1	+8.3
G.W.	57	"	1	6.95	8	870	0.91	44.2	39.2	+5.0		
U.B.	59	" " toxic.	1	8.60	33	260	0.76	31.6	21.1	+10.5		
J.J.G.	60	Addison's disease.	1	6.85	14	490	0.74	38.5	26.5	+12.0		
E.N.S.	66	Mediastinal tumor.	1	7.80	15	520	0.86	40.6	30.7	+9.9		
J.P.	40	Lead poisoning, parathyroid therapy.	1	3.80	7	540	0.77	44.6	35.0	+9.6		
Whole series.....										+5.3	+12.0	+2.5
Normal.....										+4.7	+8.4	+2.5
Pathological.....										+8.5	+12.0	+2.8

Immediately preceding or following the automatic sample, from 3 to 5 HP samples were collected. Each was taken at the end of a normal expiration.

Table I presents a summary of 52 such experiments. But little comment is required. In the four normal subjects, whether

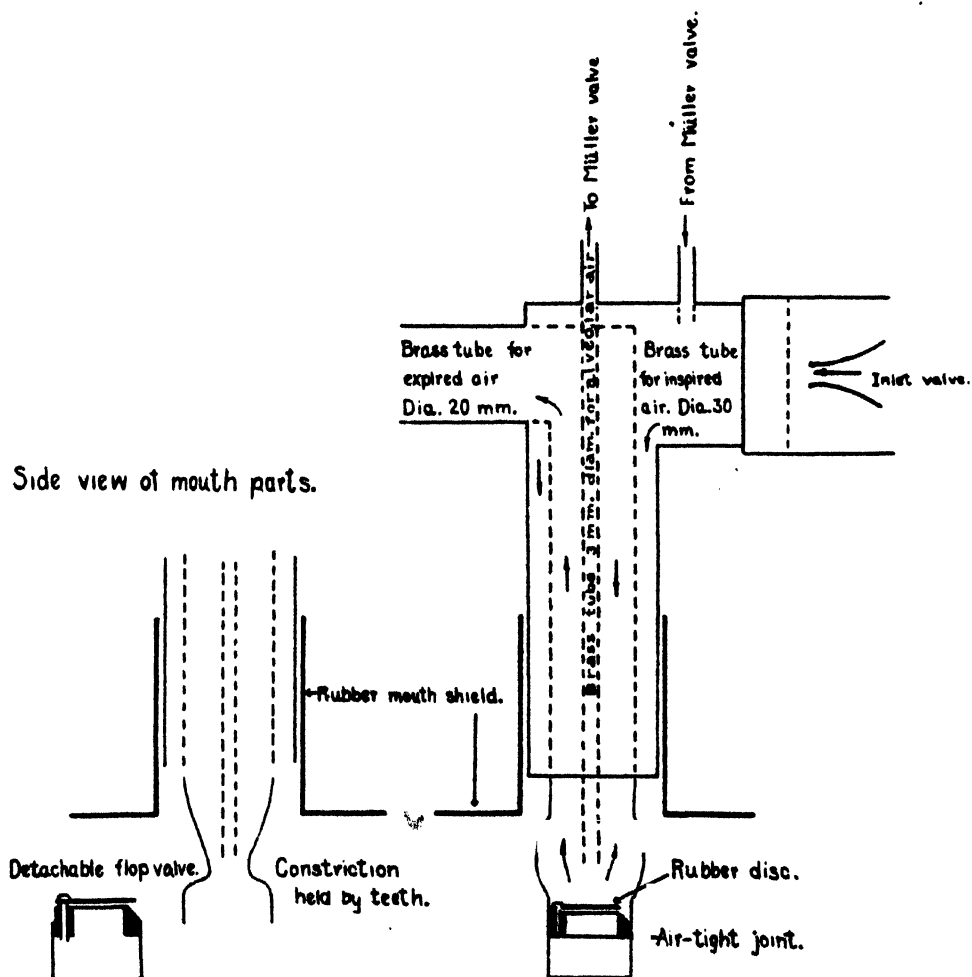


FIG. 1. A new device for automatically collecting samples of alveolar air.

basal or not, the carbon dioxide pressure of the automatic samples was 4 to 6 mm. below the true alveolar pressure. The extreme range was from 2.5 mm. below to 8.4 mm. below. With the seven pathological subjects, the differences were more divergent, ranging from 2.8 to 12.0 mm. below and averaging 8.5 mm. below

the average pressure of the HP samples. There was no clear relationship between magnitude of the discrepancy and volume of tidal air although the greatest differences were observed in pathological subjects having a small tidal air.

Henderson and Haggard have recognized that the automatic device as they have used it gives low values for the carbon dioxide pressure of alveolar air. Their correction factor is 4 per cent, about 1.6 mm. They suggest that this difference is due to overbreathing. We have included in Table I the respiratory quotients for basal subjects. For the most part these were within the normal range, which leads us to think this explanation an improbable one.

It seemed likely to us that the large dead space of the HH valve and the large volume of air that must be flushed out was responsible for these low results. While our experiments were under way, Moore, Hamilton, and Kinsman (3), by reducing the dead space of the valve, obtained results approaching those of the HP method. Cordero (4), by reducing the dead space of the valve and by omitting the large ethyl iodide sampling tube, also obtained values similar to those of the HP method.

We have proceeded to make a simultaneous study of several methods of collecting alveolar air automatically, using as a criterion the average pressure of several HP samples, each collected at the end of expiration. One of us (L.M.H.) designed an automatic valve, Fig. 1, similar in some respects to Cordero's and having no dead space, the valve being within the mouth. The HH valve was used also, both with and without the interposed sampling tube. Each of these four methods was employed in turn repeatedly on each of several subjects all of whom were male laboratory workers, who, with the exception of T. C., were experienced in respiratory technique. The subjects were reclining but were not in the basal state. The results of three typical experiments, including ventilation uncorrected to standard conditions, respiratory rate, and tidal air are given in detail in Table II. The data are recorded in the order of experimental procedure. The results of these and several additional experiments on other subjects are summarized in Table III.

TABLE II.

*Comparison of Haldane-Priestley with Three Automatic Methods of Collecting Alveolar Air.*

Three detailed experiments on resting subjects, each in reclining position throughout the experiment.

Method.	Average ventilation.	Respiratory rate per min.	Average tidal air.	Period.	Alveolar $p\text{CO}_2$ .
A. Subject T.C.					
	<i>liters per min.</i>		<i>cc.</i>	<i>min.</i>	<i>mm. Hg</i>
HP.	7.21	18	400		40.6 41.1 40.8 40.8 <u>40.8</u> Average.
New valve.	7.18 6.70 8.22	23 22 20	312 304 411	5 7 3	38.0 39.0 37.9 <u>38.3</u> Average.
HP.					39.9 40.2 <u>40.1</u> Average.
HH valve.	7.64 7.02	18 18	422 390	5 5	34.1 35.5 <u>34.8</u> Average.
HH valve; 250 cc. tube interposed.	8.36	17	491	15	32.5
New valve.	6.21	19	327	5 5	38.3 39.1 <u>38.7</u> Average.
HP.					40.4 39.6 41.1 <u>40.4</u> Average.
B. Subject D.B.D.					
HP.	5.42	10	542		42.3 41.8 42.2 43.3 <u>42.4</u> Average.

TABLE II—*Continued.*

Method.	Average ventila- tion.	Respira- tory rate per min.	Average tidal air.	Period.	Alveolar pCO <sub>2</sub> .
B. Subject D.B.D.— <i>Continued.</i>					
	<i>liters per min.</i>		<i>cc.</i>	<i>min.</i>	<i>mm. Hg</i>
New valve.	5.75	10	575	5	39.2
				10	38.9
				5	39.5
				15	39.3
					39.3 Average.
HP.	5.86	10	586		41.6
					42.8
					41.7
					42.1
					42.1 Average.
HH valve.	5.78	10	578	5	35.3
	5.83	11	524	10	39.9
	5.56	10	556	5	38.0
	5.80	11	527	15	36.2
					37.4 Average.
HH valve; 250 cc. tube interposed.	5.72	10	572	10	32.8
				15	35.2
					34.0 Average.
					42.0
					42.5
					42.4
					43.2
					42.5 Average.
C. Subject A.V.B.					
HH valve.	5.61	5	1120	5	37.8
	5.94	5	1187	5	37.5
					37.7 Average.
HP.	4.88	4	1220		39.2
					39.4
					39.3 Average.
New valve.	4.94	4	1235	7	37.5
	5.07	4	1267	7	36.2
					36.8 Average.

TABLE II—*Concluded.*

Method.	Average ventila- tion.	Respira- tory rate per min.	Average tidal air.	Period.	Alveolar $p\text{CO}_2$
C. Subject A.V.B.— <i>Continued.</i>					
	<i>liters per min.</i>		<i>cc.</i>	<i>min.</i>	<i>mm. Hg</i>
HH valve.	5.48	4	1367	5	36.8
HH valve; 250 cc. tube interposed.	6.08	4	1520	15	32.5
HP.					38.8 39.4 39.1 Average.

TABLE III.

*Comparison of Carbon Dioxide Pressure of Haldane-Priestley Samples with  
That of Samples Collected by Automatic Methods.*

Summary of experiments on eight individuals, each in reclining position  
throughout the experiment.

Subject.	Method of collecting alveolar samples.				$\Delta p\text{CO}_2$ , taking HP method as standard.		
	HP.	New valve.	HH valve.	HH valve with 250 cc. tube inter- posed.	New valve.	HH valve.	HH valve with 250 cc. tube inter- posed.
	<i>mm. Hg</i>	<i>mm. Hg</i>	<i>mm. Hg</i>	<i>mm. Hg</i>	<i>mm. Hg</i>	<i>mm. Hg</i>	<i>mm. Hg</i>
D.B.D.....	42.3	39.2	37.4	34.0	-3.1	-4.9	-8.3
T.C.....	41.3	38.95	34.0		-2.35	-7.3	
D.B.D.....	40.7	40.0			-0.7		
A.V.B.....	39.8	41.15	38.9	34.7	+1.35	-0.9	-5.1
L.A.O.....	40.5	39.6	38.5	38.0	-0.9	-2.0	-2.5
C.V.C.....	42.8	40.65	35.4	33.3	-2.15	-7.4	-9.5
A.V.B.....	39.15	36.8	37.2	32.5	-2.35	-1.95	-6.65
T.C.....	40.4	38.1	34.8	32.5	-2.3	-5.6	-7.9
Average.....					-1.6	-4.3	-6.6

## DISCUSSION.

Evidently, in any automatic device for collecting true alveolar air, the dead space must be reduced to a minimum. Our new valve gives samples having from 1 to 3 mm. lower pressure than HP samples collected at the end of normal expiration. This small discrepancy persists whether the tidal air is less than 400 cc. as in the case of T.C. or over 1000 cc. as in the case of A.V.B. We have no evidence that one magnitude is a function of the other. It is clear that failure to wash out the dead space cannot be an explanation of this difference.

Haldane (5) has shown that the percentage of carbon dioxide in the last fraction of a forced expiration does not vary as the volume expired is increased beyond 800 cc. We have made a preliminary study of the carbon dioxide pressure throughout the respiratory cycle in resting subjects. The subject expires through a rubber tube of about 2 cm. inside diameter and 100 cm. long into a vital capacity spirometer. Three factors were varied at will; *viz.*, the point in the respiratory cycle when the signal "blow" was given, the time employed in the forced expiration, and the total volume expired. These preliminary experiments leave us in accord with the above mentioned observation of Haldane, provided the time employed in the forced expiration is kept constant. When the time is carefully controlled, it can be shown that the dead space is completely flushed out with an expiration not exceeding 400 cc. If one collects a series of HP samples in which (a) the volume expired is kept constant and in excess of 400 cc., (b) the signal is given in the same phase in the respiratory cycle but (c) the period required for completing the expiration is varied from 1 to 15 seconds, it will be found that the carbon dioxide pressure of the last fraction will increase from 1 to 2 mm. per second until the venous pressure is approached. Krogh and Lindhard (6) have pointed out this source of error in the HP method during exercise when carbon dioxide production is rapid but concluded that the error during rest was not great. These preliminary observations made on the changes in carbon dioxide pressure in the respiratory cycle convince us that the HP sample collected at the end of normal expiration is higher than any of the automatic samples simply because the last fraction of the HP sample comes from a later phase in the respiratory cycle than any of the automatic samples.

At the present time knowledge of the diffusion cycle of gases in the lung is quite incomplete. We do not know, for example, at what point in the respiratory cycle the pressure of carbon dioxide in the alveoli most nearly approaches the average carbon dioxide pressure in the arterial blood. Nor in fact do we know that it is the same phase in the cycle for every individual. We have the experimental demonstration, however, that HP samples at the end of expiration have, for most resting individuals, approximately the same carbon dioxide pressure as arterial blood. The automatic methods used give distinctly lower results than the HP method and we conclude that the latter method yields the most reliable data concerning the state of the arterial blood during a complete respiratory cycle while the subject is at rest.

#### SUMMARY.

It has been shown that the automatic sampling device used in the determination of the rate of blood flow by the ethyl iodide method gives values much too low for the carbon dioxide pressure of arterial blood.

Even when the dead space of the automatic valve is reduced to a minimum, or eliminated entirely, the carbon dioxide pressure of such samples is 1 to 3 mm. less than the Haldane-Priestley samples. In resting subjects, the HP method gives the most reliable expression of the state of the arterial blood with respect to the carbon dioxide pressure.

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# THE CARBON DIOXIDE EQUILIBRIUM IN ALVEOLAR AIR AND ARTERIAL BLOOD.

## III. EXERCISING SUBJECTS.\*

BY D. B. DILL,† J. S. LAWRENCE,‡ L. M. HURXTHAL,  
AND A. V. BOCK.

*(From the Medical Laboratories of the Massachusetts General Hospital,  
Boston.)*

(Received for publication, June 6, 1927.)

It is a familiar thesis that if the diffusing apparatus of the lung permits 95 per cent saturation of the blood with oxygen, substantial equilibrium between the carbon dioxide pressures on opposite sides of the alveolar membrane will be attained. The principal supporting argument is the fact that carbon dioxide diffuses 20 to 30 times as rapidly as oxygen. It is assumed that the transfer of each gas is a diffusion phenomenon. That such an equilibrium exists in resting subjects has been demonstrated experimentally by Bock and Field (1) and Dautrebande (2).

Krogh and Lindhard (3) have studied the changes in alveolar carbon dioxide pressure in exercise, but, in common with many others, doubt the accuracy with which such determinations portray the arterial carbon dioxide pressure. It has been our purpose to investigate the possibility of collecting, during exercise, alveolar air which will measure the average carbon dioxide pressure of arterial blood.

\* The expenses of this research were defrayed in part by the Tutorial Fund of Harvard University.

A preliminary report of this research was presented to the Society for Clinical Investigation at Atlantic City, May 3, 1926.\*

† National Research Fellow in Chemistry.

‡ Edward Hickling Bradford Fellow in Medical Research, Harvard Medical School.

## EXPERIMENTAL.

In each of these experiments, unless otherwise noted, the subject rode at a constant speed on a stationary bicycle. By suitable adjustment of the brake, any metabolic level up to 10 times the basal rate could be reached. The apparatus was arranged so that the subject inspired outdoor air and expired through a mixing chamber of 7 liters capacity into a calibrated spirometer. Samples of expired air for analysis could be drawn from the mixing chamber as often as desired. The ventilation rate could be determined by reading the spirometer gauge at intervals of 1 minute or longer. At a convenient time, usually after a steady state had been reached, arterial blood was withdrawn under novocaine anesthesia from the radial or the brachial artery. During its withdrawal, samples of alveolar air were taken by the Haldane-Priestley method. In a few experiments, additional alveolar samples were collected automatically, by use of the Müller valve as described by Henderson and Haggard (4).

The pressure of carbon dioxide in arterial blood was determined as previously described (1). The volume and composition of the air expired during a measured period immediately preceding or following the withdrawal of the blood established the metabolic level.

In the early experiments, the Haldane-Priestley samples were collected by giving the instruction "blow" at the end of a normal expiration. Bock and Field (1) had found that for resting subjects samples collected in this manner were in approximate carbon dioxide equilibrium with arterial blood. The results of our early exercise experiments indicated that at high metabolic levels there is a slight piling up of carbon dioxide during the delay incident to this method of sampling, as suggested by Krogh and Lindhard (3). Experiments were then undertaken in which the instruction "blow" came as the subject began his expiration. Such samples were found to have essentially the same carbon dioxide pressure as that of arterial blood. This method was adopted, therefore, for all later experiments.

In the course of this investigation, twenty-two arterial punctures were made. In twenty of these experiments, samples of alveolar air were collected and the equilibration of the blood was carried to a satisfactory conclusion. Accidents prevented the comple-

TABLE I.

*Carbon Dioxide Pressure of Arterial Blood and of Alveolar Air at Various Metabolic Levels.*

Experi- ment No.	Subject.	Date.	Oxygen used per min.	Arterial blood $p\text{CO}_2$ .	Alve- olar air $p\text{CO}_2$ .	Blood - air $p\text{CO}_2$ .	Remarks.
		1926	cc.	mm.	mm.	mm.	
1	A.V.B.	Jan. 13	1480	38.8	38.8	0.0	
2	"	" 19	1250	38.0	39.7	-1.7	
3	"	" 26	1660	35.2	39.4	-4.2	
4	"	Feb. 3	1440	38.8	38.5	+0.3	
5	"	" 17	1770	35.4	36.4	-1.0	
6	"	Mar. 26	800	37.0	36.1	+0.9	3rd minute after stopping work.
7	"	Apr. 6	1230	39.4	38.4	+1.0	$p\text{CO}_2$ by automatic valve, 39.4 mm.
8	D.B.D.	Jan. 15	1710	47.6	48.1	-0.5	
9	"	Feb. 11	1730	41.0	40.9	+0.1	
10	"	" 26	2110	42.7	44.5	-1.8	4th minute after work began.
11	"	Mar. 23	2100	43.3	43.9	-0.6	$p\text{CO}_2$ by automatic valve, 44.3 mm.
12	"	Apr. 14	2200	44.7	44.1	+0.6	
13	J.S.L.	Feb. 12	250	42.7	43.1	-0.4	Resting.
14	"	Apr. 8	600	33.0	32.5	+0.5	3rd and 4th minutes after stopping work.
15	L.M.H.	Jan. 8	360	40.0	39.0	+1.0	Sitting on bicycle.
16	"	" 8	800	43.8	44.9	-1.1	2nd and 3rd min- utes after work began.
17	"	Feb. 9	1720	38.1	42.2	-4.1	
18	"	Mar. 19	1540	42.5	41.9	+0.6	$p\text{CO}_2$ by automatic valve, 41.8 mm.
19	C.P.	" 25	1500	39.2	43.1	-3.9	Inexperienced sub- ject; $p\text{CO}_2$ by automatic valve. 42.3 mm.
20	F.A.	Apr. 27	1400	43.0	46.4	-3.4	Inexperienced sub- ject.

tion of the other two experiments. The results of these twenty experiments are summarized in Table I.

Sixteen of these twenty experiments were made on trained sub-

jects during exercise. The results of the sixteen experiments are arranged in two series in Table II. In the seven experiments of the first series, the samples of air were collected at the end of expiration. In the other series, they were collected at the beginning of expiration.

In Table III, is found the complete record of a single exercise experiment in which no arterial puncture was made. Alveolar air was collected at the beginning and at the end of expiration as well

TABLE II.

*Haldane-Priestley Samples of Alveolar Air in Exercise. Comparison of Samples Collected after Expiration with Samples Collected at Beginning of Expiration.*

Two series of experiments on four experienced subjects.

After expiration.			Beginning of expiration.		
Subject.	Date.	Blood - air $p\text{CO}_2$ .	Subject.	Date.	Blood - air $p\text{CO}_2$ .
	1926	mm.		1926	mm.
L.M.H.	Jan. 8	-1.1	D.B.D.	Feb. 11	+0.1
A.V.B.	" 13	0.0	A.V.B.	" 17	-1.0
D.B.D.	" 15	-0.5	D.B.D.	" 26	-1.8
A.V.B.	" 19	-1.7	L.M.H.	Mar. 19	+0.6
"	" 26	-4.2	D.B.D.	" 23	-0.6
"	Feb. 3	+0.3	A.V.B.	" 26	+0.9
L.M.H.	" 9	-4.1	"	Apr. 6	+1.0
			J.S.L.	" 8	+0.5
			D.B.D.	" 14	+0.6
Average.....		-1.6	Average.....		0.0

as by the automatic method of Henderson and Haggard (4). A summary of this and thirteen similar experiments appear in Table IV.

#### DISCUSSION OF RESULTS.

In all but four of the experiments in Table I, fair agreement existed between the carbon dioxide pressure of arterial blood and that of alveolar air. The difference in pressure did not exceed 1 mm. in twelve cases.

These experiments covered a metabolic range up to 10 times the

basal rate. Evidently, in exercise of this grade, it is possible to collect samples of alveolar air which have essentially the same carbon dioxide pressure as that of arterial blood. It is interesting to note that in the four experiments in which the automatic method was used, the results checked the HP<sup>1</sup> method.

The changes observed in the alveolar carbon dioxide pressure in exercise are similar to those recorded by other investigators.

TABLE III.

*Comparison of Carbon Dioxide Pressures of Haldane-Priestley with Henderson-Haggard Alveolar Air Samples in Exercise.*

Summary of fourteen experiments on five normal subjects.

Subject.	Date.	Oxygen used per min.	Respi- rations per min.	Tidal air.	HP samples at beginning of expiration.		HH samples.		$p\text{CO}_2$ HP - $p\text{CO}_2$ HH.
					No. of sam- ples.	Aver- age $p\text{CO}_2$ .	No. of sam- ples.	Aver- age $p\text{CO}_2$ .	
	1925	cc.		cc.		mm.		mm.	mm.
A.V.B.	Apr. 6	1200	20	1500	5	37.9	1	39.4	-1.5
D.B.D.	Mar. 15	1300	18	1500	5	43.6	7	43.4	+0.2
	" 17	1700	20	1600	4	42.3	4	42.5	-0.2
	" 17	1700	20	1600	4	40.1	4	41.5	-1.4
	" 17	1700	20	1600	4	39.8	3	42.2	-2.4
	" 22	900	15	1300	3	41.4	2	42.0	-0.6
	" 23	2100	23	1900	2	43.9	2	44.3	-0.4
	Apr. 6	1900	21	1800	4	42.8	4	42.1	+0.7
L.M.H.	Mar. 17	1700	16	2100	4	42.6	4	39.3	+3.3
	" 17	1700	16	2100	4	40.0	4	39.9	+0.1
	" 19	1500	15	1900	4	41.9	1	41.8	+0.1
	Apr. 1	2800	23	2600	3	41.6	1	39.7	+1.9
C.P.	Mar. 25	1500			3	43.1	2	42.3	+0.8
G.C.C.	" 31	1800			2	47.0	1	47.3	-0.3

After a steady state is reached, the carbon dioxide pressure is not far from the resting level. There is often an abrupt drop after stopping work. This is illustrated by Experiment 14; both blood and air reached a level of about 32 mm., which was about 6 mm. below the exercise level.

It is curious that the last two experiments in Table I showed

<sup>1</sup> HP is used to designate the Haldane-Priestley method; HH, the Henderson-Haggard valve method.

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such unsatisfactory results. We are inclined to attribute this to the inexperience of the subjects. Each was somewhat agitated during the withdrawal of the blood. Upon hearing the signal "blow," F. A. increased his speed considerably. However, the fact that the HH and the HP samples of Experiment 19 checked,

TABLE IV.

*Comparison of Carbon Dioxide Pressures of Haldane-Priestley with Hender-son-Haggard Alveolar Air Samples in Exercise.*

Representative experiment on D.B.D. while using 1300 cc. of oxygen per minute.

Time.	HP samples.		HH samples.	Respirations per min.	Pulse.
	End of expiration.	Beginning of expiration.			
<i>min.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>		
9-11			42.9	18	112
16	46.8				
18-20			44.6	18	112
21		43.0			
23	45.1				
24-26			44.5		
27	46.5				
28		44.7			
31-33			42.8	20	114
35		44.4			
40-42			43.7		114
43		43.1			
45	43.5				
47-49			42.3		
50		43.1			
52	44.0				
53-55			43.2		114
Average.....	45.2	43.6	43.4		

suggests that here there may have been an error in determining the carbon dioxide pressure of the blood.

When the results on experienced, exercising subjects are classified according to the method of collecting the alveolar samples (Table II), it is seen that the HP samples collected at the beginning of expiration show but one discordant figure. The average carbon

dioxide pressure of the blood in this series is precisely that of the alveolar air. The two most discordant results came in the early group when we were collecting the samples of alveolar air at the end of expiration. We were then less experienced; hence it is not possible to ascribe these irregularities solely to the method of sampling.

The several experiments of Tables III and IV were undertaken in the light of our accumulated experience. They establish the difference between alveolar airs collected by different methods from exercising subjects. The typical experiment of Table III makes it clear that samples collected at the end of expiration have an appreciably greater pressure than samples collected at the beginning of expiration. Those collected at the beginning of expiration check those obtained automatically.

During exercise, then, the automatic device of Henderson and Haggard gives an accurate measure of the carbon dioxide pressure of the arterial blood. Its ability to do so, despite its failure with resting subjects is quite likely due to the fact that the HP samples collected during exercise at the beginning of expiration come from the same phase in the respiratory cycle as the HH automatic samples.

In view of these results it does not appear necessary to utilize the volume of the dead space to obtain the pressure of  $\text{CO}_2$  in alveolar air during exercise as suggested by Krogh and Lindhard (3). Owing to the rapidly changing pressure of carbon dioxide in the lungs during exercise, it is in the nature of fortuitous chance that experimental procedures yield as good results as can be obtained with respect to the equilibrium of carbon dioxide in arterial blood and pulmonary air. The data above cover a range of moderate exercise only.

#### SUMMARY.

It has been shown that Haldane-Priestley samples of alveolar air collected during exercise at the beginning of expiration measure approximately the average carbon dioxide pressure of arterial blood. Samples collected by the Henderson and Haggard automatic method are similar, but Haldane-Priestley samples collected at the end of expiration are more divergent and tend to give higher values.



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# EFFECT OF ANTIRACHITIC VITAMIN ON THE PHOSPHORUS, CALCIUM, AND pH IN THE INTESTINAL TRACT.\*

By LESTER YODER.

*(From the Chemistry Section of the Agricultural Experiment Station,  
Iowa State College, Ames.)*

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## INTRODUCTION.

In a previous article (1) it was stated that the problem of calcium equilibrium in animal nutrition in which this Station has been interested, fundamentally involves antirachitic vitamin. A new project is now under way in which an explanation of the action and nature of this vitamin is being sought.

## HISTORICAL.

Considerable work has been reported on this subject from the standpoint of blood chemistry and calcification. Since an evidence of vitamin D deficiency is abnormal bone development, we find a number of investigations dealing with the deposition of calcium and phosphorus. Holt, La Mer, and Chown (2) developed a mechanism of calcification. They found that calcium and phosphorus in the form of phosphates can exist in a medium such as the blood in supersaturated solution and that the deposition of these elements to form bone can only take place when such supersaturation exists in the blood. It was also shown that the lower the alkalinity the greater is the ion product of calcium and phosphate ions in the equilibrium,

$$\frac{[\text{Ca}]^{++} \times [\text{PO}_4]^{=}}{[\text{Ca}_3(\text{PO}_4)_2]} = K_{s.p.}$$

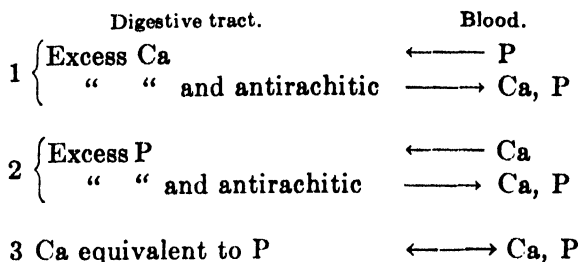
and the concentration, therefore, of diffusible calcium and phosphorus.

There is a number of investigations dealing with the changes in the composition of the blood with respect to calcium and phosphorus during the

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onset and cure of rickets. The work of Howland and Kramer (3), Salvesen, Hastings, and McIntosh (4), and Park, Guy, and Powers (5), shows that for normal calcium and phosphorus retention in the prevention or cure of rickets there should be no excess of either in the digestive tract in the absence of antirachitic vitamin. The interpretation of their work in the three possible conditions can best be shown diagrammatically, thus:



Shipley and coworkers (6) established the significance of the calcium and phosphorus ratio in the etiology of rickets. Bethke, Steenbock, and Nelson (7) obtained growth on a ration low in calcium by addition of either calcium or cod liver oil.

From the work of Holt (2) and coworkers and others we learn that an increase in the alkalinity or pH of a solution, containing calcium and phosphorus ions, can easily remove one or the other of these ions from solution and subsequent absorption, depending upon which is in excess. A summary of all the relations of importance in the development of this subject so far can be very well depicted by the hypothetical curve shown in Fig. 1. It would appear therefore that the factor conducive to rickets is deficient absorption of calcium and phosphorus from the intestinal tract.

These results coincided with the discovery of Zucker (8) that rickets could actually be prevented by substituting for calcium lactate in a rachitic ration, calcium chloride or ammonium chloride, two salts which are more acid than the lactate. Shohl and Sato (9) found that defective absorption of calcium and phosphorus was due to alkalinity. Blum, Delaville, and Caulaert (10) concluded that the origin of rickets was due to a modification of the physicochemical state of the mineral content of the intestinal tract by an alteration in the acid-base equilibrium. Irving and Ferguson (11) found a much more rapid absorption of calcium by the blood from solutions of calcium chloride buffered at pH 3.0 than at pH 8.0 in the intestines of anesthetized dogs. Jones (12) caused rickets to develop in puppies fed an adequate diet but containing high potential alkalinity and was able to cure rickets in children by the addition of hydrochloric acid to their diet.

More recently Zucker and his coworkers (13) have found that the administration of antirachitics and irradiation with ultra-violet light lowered the pH of the water suspension of the feces on the average from 7.6 to 6.2. According to the work of McClendon and coworkers (14), the lower portions of the digestive tract should be more alkaline, and antirachitics and ultra-violet light, therefore, should yield a more acid medium in the upper

portions of the tract where much of the absorption has been shown to take place.

Very recently Abrahamson and Miller (15) reported that the pH of the intestinal contents of rats fed antirachitic diets varied from 5.2 to 6.5. The pH became 6.4 to 7.4 when the Pappenheimer-Sherman (16) rachitic diet was used. The addition of cod liver oil to this diet lowered the pH values to normal. The conclusion drawn was that the rise in pH with the resulting formation of insoluble calcium phosphate in the intestine is probably an important factor in deficient calcium absorption in rickets.

There is evidence, therefore, that substances or processes that are antirachitic effect a decrease in pH in the intestinal tract. It was the purpose of the work reported in this article to establish

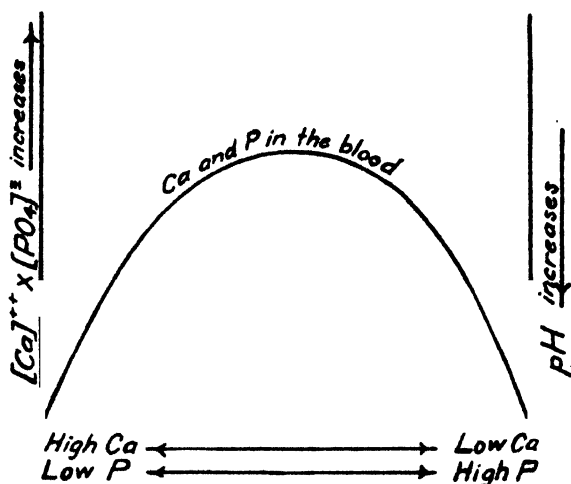


FIG. 1. Relation between pH and calcium and phosphorus concentration in the intestinal tract and in the blood.

by means of an experiment with rats the relation of antirachitic vitamin to the pH of the intestinal contents and to the movement of calcium and phosphorus into or out of the digestive tract.

#### EXPERIMENTAL.

Male albino rats, weighing 150 gm., in individual cages with raised screen bottoms were fed the rachitic ration consisting of 76 parts of yellow corn, 20 parts of gluten, 3 parts of calcium carbonate, and 1 part of salt. In order that the vitamin effect preceding a rachitic condition could be studied, observations were made which showed at 21 days a general decrease in phosphorus

utilization and a constant positive utilization of calcium. Two rats were then fed the ration and 4 per cent cod liver oil, two others were fed the ration and irradiated 10 minutes daily, and three more were kept on the rickets ration for controls. At the end of 30 days when feed and fecal analysis showed distinct differences in the absorption of calcium and phosphorus by the three groups, three rats, one from each group, were chloroformed and the contents of the intestinal tract examined for pH.

### *I. Calcium and Phosphorus Utilization.*

Recent publications by Bergeim (17) on intestinal chemistry have demonstrated that the nature of the carbohydrate portion of the diet of rats has an important effect on calcium and phosphorus absorption. The movement of these elements through the walls of the gut was studied by him when the animals were on a rachitic diet and when they were on such a diet supplemented with cod liver oil.

The chemical methods used by Bergeim to determine the utilization of calcium and phosphorus were based on corresponding changes in the ratio of the substance studied to the iron in the feed and in the intestinal medium. These methods were admirably adapted to this experiment where much depended upon the ability to make many accurate determinations in a short time. They were used without modification and are therefore not described in this article.

1 per cent of hydrated ferric hydroxide powder was incorporated in the rachitic diet and the mixture ground to pass a 30 mesh sieve. Ratios of mg. of calcium to mg. of iron and mg. of phosphorus to mg. of iron per cc. of the hydrochloric acid solution of the ash of a small portion of this feed were determined. Ratios for the same elements in the feces were determined and the percentage utilization calculated from the formula:

$$100 - \frac{\left[ \frac{\text{Ca or P}}{\text{Fe}} \right]_{\text{feres}}}{\left[ \frac{\text{Ca or P}}{\text{Fe}} \right]_{\text{feed}}}$$

In Table I are recorded the percentages of feed calcium and phosphorus utilized by the animals after 15, 21, 25, 28, and 30 days. The means of these percentages are also included for the

rats receiving the rickets ration and cod liver oil, for those receiving the rachitic ration and irradiation, and finally for the control rats on the rachitic ration alone. The significance of these figures is shown by the graphs in Fig. 2.

TABLE I.  
*Percentage Utilization of Feed Calcium and Phosphorus.*

Ration.	Rat No.	15 days.	21 days.	25 days.	28 days.	30 days.
Calcium.						
Rickets ration and cod liver oil.	1	2	-19	-40	-19	-34
	2	11	35	-25	23	-8
Mean.....		9	8	-32	2	-21
Rickets ration and irradiation.	3	4	12	-19	5	-37
	4	2	-3	-91	9	-41
Mean.....		3	4	-55	7	-39
Rickets ration.	5	15	18	-12	13	-112
	6	18	17	-61	-46	-130
	7	21	6	-32	-4	-93
Mean.....		18	21	-35	-18	-112
Phosphorus.						
Rickets ration and cod liver oil.	1	-2	-53	-108	-88	-9
	2	+3	-12	-47	-47	+9
Mean.....		0	-33	-78	-68	0
Rickets ration and irradiation.	3	-38	-11	-58	-38	-19
	4	-30	-57	-150	-65	-30
Mean.....		-34	-34	-104	-52	-25
Rickets ration.	5	-10	-20	-27	-33	-82
	6	+1	-67	-107	-115	-111
	7	-7	-4	-17	-79	-55
Mean.....		-5	-30	-50	-76	-83

## II. pH of Intestinal Contents.

An important point in establishing a theoretical mechanism for the action of antirachitic vitamin rests on the relation between the

hydrogen ion concentration of the alimentary medium and calcium and phosphorus utilization as influenced by the antirachitic agents used.

The rats were chloroformed individually and the intestinal tract removed at once. The duodenum and ileum were cut into a dozen or more sections and samples obtained by pressure on a representative number of pieces. The pH was determined by means of the quinhydrone electrode (18), first in the fresh feces

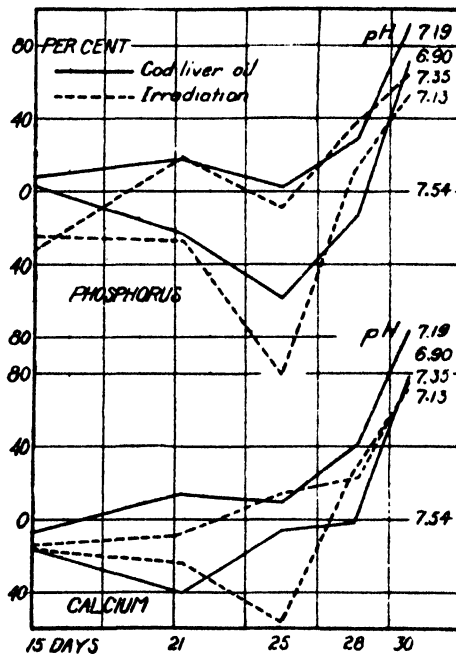


FIG. 2. Effect of cod liver oil and irradiation on the variation of the per cent utilization of phosphorus and calcium from the means for rats on a rachitic ration.

of the living rat, then at once in the contents of the duodenum, the ileum, and the cecum of the dissected rat.

The pH figures for each animal are recorded in Table II together with their means for the animals receiving the same diet or treatment.

#### DISCUSSION.

Large rats were used in order that sufficient samples from the intestinal tract might be available for examination. Older animals are also not as susceptible to acute forms of rachitic condi-

tions. None of the rats showed symptoms of rickets. Therefore they had not reached a stage where they presented an exaggerated rachitic relation between calcium and phosphorus utilization and intestinal pH. In general, the results obtained show that important effects are brought about even in more mature animals by the addition of antirachitic vitamin to a rachitic diet.

The figures for calcium and phosphorus utilization vary greatly among individual animals. For the animals kept on the rachitic diet, the means show a steady decrease in the utilization of phosphorus and an irregular decrease in calcium utilization. Addition of cod liver oil to this diet and irradiation of the animals first

TABLE II.  
*pH of Feces and Contents of Duodenum, Ileum, and Cecum.*

Ration.	Rat No.	Duode-num.	Ileum.	Cecum.	Feces.	Mean.
Rickets ration and cod liver oil.	1	6.27	6.75	7.48	7.12	6.90
	2	6.66	7.05	7.63	7.43	7.19
Mean.....		6.46	6.90	7.55	7.27	7.04
Rickets ration and irradiation.	3	7.00	7.00	7.86	7.55	7.35
	4	6.74	6.71	7.43	7.64	7.13
Mean.....		6.87	6.85	7.64	7.59	7.24
Rickets ration.	5	6.75	7.06	8.50	8.00	7.58
	6	6.77	7.28	7.97	7.71	7.43
	7	6.66	7.83	7.95	7.98	7.60
Mean.....		6.72	7.39	8.14	7.90	7.54

caused a decreased utilization of phosphorus, then very important increases in utilization of both calcium and phosphorus at the end of the experiment.

The preliminary decrease in phosphorus utilization is significant. Considered in the light of results reported by Bergeim (17) in which phosphorus excretion into the upper tract occurred when much calcium was absorbed, it is an indication that cod liver oil and particularly irradiation cause excretion of phosphorus into the intestine. Apparently, the reabsorption from the lower tract, noted as an effect of cod liver oil with a rachitic diet by Bergeim,



did not take place until vitamin D became effective at the close of the experiment.

If phosphorus is excreted into the upper tract as phosphate ion the reaction in the small intestine should be a lowered pH or increased acidity due to a content of phosphoric acid. The irradiation data show a low pH beyond the duodenum only, a variation in reaction which is characteristic for irradiated animals on a rachitic ration. It is surely an evidence of the excretion of an acid or an acid-producing substance into the small intestine due to the action of radiant energy. These are the only data that we know of which support a distinction between the action of the antirachitic agents, cod liver oil and ultra-violet irradiation.

The pH results show complete agreement with the work of Zucker (13) and Abrahamson (15) who found the effect of antirachitics on the reaction of the feces and the intestinal contents to be a lowered pH. There is complete agreement with the theory that alkalinity in the intestinal tract is a cause of poor calcium and phosphorus utilization and that acidity favors the utilization and prevents loss of these elements from the body and the consequent rachitic condition of the animals. An examination of Fig. 2 which shows the variation of per cent utilization of calcium and phosphorus from the mean for the rats on the rachitic diet and its relation to the average pH of the digestive tract as affected by cod liver oil and irradiation, shows that the pH test proposed by Zucker (13) for antirachitic vitamin should be reliable.

#### SUMMARY.

1. In rats fed a rachitic ration calcium utilization from the intestinal tract changed from a small absorption to a relatively large elimination. Phosphorus utilization from the intestinal tract of these rats changed at the same time from a slight elimination to a relatively large elimination.

2. Irradiation of rats on a rachitic ration or addition of cod liver oil to such a ration, decreased the elimination of calcium through the intestinal tract, and ultimately the elimination of phosphorus.

3. A preliminary effect of irradiation of rats on a rachitic ration was an increase in phosphorus elimination from the intestinal

tract. Cod liver oil in a rachitic ration had a similar but smaller effect.

4. A later effect of irradiation of rats fed a rachitic ration and of cod liver oil in such a ration was a decrease in the elimination of phosphorus through the intestinal tract.

5. Cod liver oil lowered the pH throughout the intestinal tract of rats on a rachitic ration. Irradiation lowered the pH of the intestinal tract beyond the duodenum only.

6. There was correlation between lowered pH and calcium and phosphorus utilization.

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## THE PREPARATION OF BORNEOL GLYCURONIC ACID AND GLYCURONIC ACID.

By ARMAND J. QUICK.

*(From the Department of Surgical Research, Cornell University Medical  
College, New York City.)*

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The significance of glycuronic acid in the economy of the organism is not fully understood, but it seems rather certain that its biological importance is underestimated. There can be no doubt that it plays a prominent part in the mechanism of detoxication in the body. Like hippuric acid and the ethereal sulfates, the conjugated glycuronic acids appear to be constant constituents of normal human urine as indicated by the slight levotation which is nearly always observed and by the fact that glycuronic acid has been isolated from urine in the form of the *p*-bromophenylhydrazine derivative (1, 2). Glycuronic acid is conjugated both with phenolic types of compounds and with various aromatic acids such as benzoic acid and phenylacetic acid, thus complementing on the one hand the action of the ethereal sulfates and that of glycocoll on the other. A quantitative study of the curious interrelationship of these detoxication mechanisms may possibly offer a means for obtaining further information concerning the synthetic power of the various organs, especially of the liver. Little emphasis has been given to the marked ability of the organism to produce relatively large amounts of glycuronic acid without any perceptible embarrassment. As an example of this power it might be cited that a dog after eating 2 pounds of prunes excreted 11 gm. of glycuronic acid in the form of glycuronic acid monobenzoate in the course of 24 hours. Even in the human organism the amount of glycuronic acid produced for conjugation with phenolic compounds is much greater than the ethereal sulfate output. The fact that glycuronic acid is produced in response to benzoic acid, phenylacetic acid, and other phenyl substituted

aliphatic acids which resist oxidation within the body, suggests the interesting probability that a similar conjugation may occur between the fatty acids and a carbohydrate molecule in normal metabolism, but that the compounds thus formed are so readily oxidized that their existence has thus far escaped detection.

Before these various problems just mentioned can be successfully studied, it stands to reason that more ought to be known about glycuronic acid itself, especially of its metabolism. Before such a study can be made, however, it is essential to secure relatively large amounts of the pure acid, and therefore the first step in the proposed research consisted in developing a satisfactory method for preparing the acid and in finding a suitable source of material. Glycuronic acid has been isolated by a number of the earlier investigators who studied this compound, but always as the acid lactone, and it was not until 1925 that the acid itself was prepared by Ehrlich and Rehorst (3). Prior to the appearance of their publication, the author, on hydrolyzing menthol glycuronic acid obtained a small amount of substance, the crystalline structure and chemical behavior of which corresponded to the glycuronic acid described by Ehrlich and Rehorst, but further work on the compound was postponed at that time on account of other experiments which were then being carried out. The method employed by the author is in principle essentially the same as the method of Ehrlich and Rehorst, but it is considerably simpler, more time-saving, and better adapted for the preparation of large quantities of glycuronic acid. In short the process consists in hydrolyzing borneol glycuronic acid with dilute sulfuric acid, subsequent removal of the mineral acid and the borneol, and finally, direct isolation of the acid from the concentrated filtrate. It may be remarked, however, that the real difficulty encountered was not in the actual preparation of the acid, but in finding a suitable source from which it might be obtained, or in other words, the primary task in hand was to find a satisfactory glycuronogenic drug. Any substance suitable for this purpose must be relatively non-toxic, thus permitting the administration of relatively large doses over long periods of time. It should furthermore give rise to a large quantity of a conjugated glycuronic acid which can readily and fairly quantitatively be isolated from urine. Although the number of conjugated glycuronic acids which have

been qualitatively determined is very large, few are suitable for the preparation of glycuronic acid. Thierfelder (4) and other pioneer investigators of glycuronic acid employed euxanthic acid, which is a conjugated glycuronic acid obtained from the natural dyestuff, purree. Since this substance is now difficult to obtain and offers no particular advantages, it was not further considered. Menthol appears to be the only substance which has given satisfactory results. It is relatively non-toxic and its conjugated glycuronic acid can readily be separated from urine by means of ammonium sulfate. When one attempts, however, to prepare large quantities of the acid, one finds menthol not altogether ideal. Of the common laboratory animals, the rabbit seems to be the only one suited for producing menthol glycuronic acid; dogs apparently can destroy menthol and also the conjugated menthol, for on feeding either form no free menthol and only a very small amount of the conjugated compound can be recovered in the excreta. When using rabbits one is compelled to give the drug by stomach tube, and since the maximum daily production of menthol glycuronic acid per animal is not very much over 2 gm., it can readily be seen that the preparation of several hundred gm. of material, the amount desired for the present series of experiments, would become a tedious and laborious task.

In order to find a drug which was more satisfactory than menthol, a number of compounds especially of the terpene series was investigated, and it was found that borneol was by far the most suitable for the purpose at hand. It is practically an innocuous drug which can be fed to dogs in 5 gm. doses per day for a period of weeks without causing any apparent deleterious effect, and without causing the slightest loss of appetite. It can be mixed directly with the food, which is a great convenience. About 50 per cent of the borneol is excreted in the urine as borneol glycuronic acid. Attempts to increase this amount have so far yielded rather unsatisfactory results, but it is hoped that further studies will determine the fate of the unaccounted for borneol as well as throw some light on the other factors concerned in the mechanism of this conjugation. The isolation of borneol glycuronic acid from urine is exceedingly easy and simple, since it can be directly and almost quantitatively precipitated as the zinc salt. Although both Hildebrandt (5) and Fromm and Clemens

(6) prepared this salt, they apparently overlooked the possibility of utilizing it as a means of isolating borneol glycuronic acid from urine and other body fluids. Since the precipitation is carried out in acidified urine, no other insoluble salt of zinc is formed unless the urine contains uric acid which is also precipitated under these conditions. By treating the urine with lead acetate uric acid as well as pigments and other interfering substances are removed without precipitating any of the desired compound. From the clarified filtrate the zinc salt of borneol glycuronic acid can be precipitated in practically chemically pure condition. On dissolving this salt in hot 3.5 N sulfuric acid, borneol glycuronic acid with a yield of about 95 per cent is obtained. It is nearly pure, and can readily be further purified by recrystallization from hot water. In this respect it offers an advantage over menthol glycuronic acid since the purification of the latter is more difficult due to the fact that the last trace of ammonium sulfate is removed with difficulty and entails a certain loss of material.

Borneol glycuronic acid belongs to the glucoside type of conjugated glycuronic acids and therefore does not reduce Fehling's solution. Its solubility in water is about 2 parts per 100, but even slight impurities will greatly increase its solubility. Fromm and Clemens (6) state that it crystallizes with 1.5 molecules of water of crystallization, but it was found that in the carefully purified air-dried product obtained in this study the water content was somewhat variable, but approximated more nearly 1 molecule of water of crystallization. The specific rotation of the compound was  $[\alpha]_D^{20} = -48$ , whereas the value for pure *d*-borneol glycuronic acid is  $[\alpha]_D^{20} = -37$ , and for the *l*-borneol glycuronic acid  $[\alpha]_D^{20} = -69$ . It is quite evident that the preparation was a mixture of the dextro and levo forms and this can be explained by the fact that the borneol used, although labelled *d*-borneol, was really a mixture of both isomers, for the rotation of the borneol employed was  $[\alpha]_D^{20} = +5$  instead of  $[\alpha]_D^{20} = +37$ , the value for the pure dextro modification. This is however of minor importance since it has no bearing on the results reported in this paper. Like other glycuronic acids, borneol glycuronic acid is a relatively strong acid although it is somewhat weaker than either the free glycuronic acid or glycuronic acid monobenzoate. Like the corresponding menthol compound, the acid is precipitated by a half saturated

solution of ammonium sulfate. It appears that this is a common property of several conjugated glycuronic acids, for purified thymol glycuronic acid is likewise precipitated, and it seems likely that other compounds of this type will be found. As previously stated, the zinc salt is insoluble even in fairly acid solution. Zinc in a dilution of 1 to 5000 can readily be detected by the rather characteristic crystalline precipitate which forms on adding an excess of borneol glycuronic acid. Since the salts of the other common metals except cadmium are soluble in either acid or neutral solution, borneol glycuronic acid is an excellent reagent for the qualitative detection of zinc and for its separation from other metals. Since the glycuronic acid in zinc borneol glycuronate can readily be determined by any of the common quantitative sugar methods, it is hoped that the insolubility of the zinc salt will form the basis for a new quantitative method for zinc. As for the determination of borneol glycuronic acid, the method developed for menthol glycuronic acid (7) is entirely satisfactory.

While borneol glycuronic acid is especially suited for preparing glycuronic acid, it also seems promising as a satisfactory drug for the study of the synthesis of glycuronic acid in the human organism. Preliminary work on this problem indicates that a dose of several gm. can be taken with impunity and that the response of the body to conjugate it with glycuronic acid is prompt and the output of the conjugated acid larger than in the case of dogs and rabbits. Since it seems probable that the liver participates in the synthesis of glycuronic acid, it may be possible to develop from this study a new test for liver function.

The preparation of glycuronic acid from borneol glycuronic acid offers no technical difficulties. The conjugated acid is hydrolyzed by means of 0.2 N sulfuric acid. The liberated borneol is insoluble in water and can be filtered off, while the sulfuric acid can be quantitatively removed by means of barium hydroxide. On concentrating the filtrate which now contains only the desired product, glycuronic acid can be obtained as a white crystalline powder. It is not pure acid, however, but contains about 30 per cent of the acid lactone. This conversion of the acid to the lactone occurs during the hydrolysis, for the solution immediately after the hydrolysis contains practically the same ratio of acid to lactone as the crystalline product. When the hydrolysis is conducted on a



water bath, instead of the solution being boiled over a free flame, the amount of lactone formed is slightly less, but no scheme of hydrolysis was found which would yield only the pure acid. The separation of the acid from the lactone offers considerable difficulty because their solubility in the various solvents is not great enough to effect a clean cut separation. Fortunately, the lactone is slightly more soluble in alcohol than the acid, and by extracting the mixture with 95 per cent ethyl alcohol one is able to obtain a product containing over 99 per cent of the free acid. Another successful though somewhat more laborious method consists in converting the acid and the lactone to a salt, reliberating the acid in the cold, removing the inorganic salt, and finally concentrating the filtrate at a low temperature by distilling under reduced pressure. This was the principle followed by Ehrlich and Rehorst (3), for they formed the barium salt as an intermediate step in their preparation of pure glycuronic acid. For most purposes it is rarely necessary to employ the pure acid; especially is this true in physiological experiments as in these the sodium salt is apt to be used almost entirely. Even for purely chemical studies it will hardly be found essential to use the pure acid unless one is specifically studying the physical or chemical properties of pure glycuronic acid.

The determination of lactone and free acid in any given sample can be readily determined by direct titration with standard alkali when phenolphthalein is used as the indicator. The complete neutralization of the acid is indicated by the first temporary persistence of the pink color. For the titration of the lactone, the solution is heated to boiling and titrated to a permanent end-point.

The lactone, or glucuron, as it is commonly called, is the more stable form, and it can be readily prepared from the acid. When glycuronic acid is merely exposed to warm dry air, it slowly loses water and becomes converted into the lactone. On dissolving the acid in hot glacial acetic acid, the lactone crystallizes out from the cooled solution. This offers a convenient method for preparing the lactone. The compound is best purified by recrystallization from hot water. On dissolving the purified product in water, one obtains a solution which is neutral to litmus and which even on standing several days shows no sign of free acid. On refluxing a solution of the lactone about 50 per cent of the acid is regenerated,

but at the same time a certain amount of disintegration occurs as indicated by the fact that the solution becomes brown and the total titratable acidity is increased. The lactone shows a specific rotation of  $[\alpha]_D^{20} = +19$ , which agrees with the value found in the literature. Theoretically there ought to be at least two modifications of the lactone, namely the  $\alpha$ - and  $\beta$ -butylene oxide forms, and it seems probable that this product having  $[\alpha]_D^{20} = +19$  may be one of these two forms. The crystals of the lactone are very characteristic. They consist of thick monoclinic plates with bevelled edges, which present a marked contrast to the needle crystals of glycuronic acid.

Glycuronic acid is a strong organic acid ( $K < 1 \times 10^{-3}$ ). It is readily soluble in water and also slightly soluble in alcohol. In cold water it is stable, but on boiling it is partly converted to the lactone. As in the case of the lactone some decomposition also takes place. An equilibrium between the acid and lactone, as observed by Ehrlich and Rehorst, occurs on refluxing a solution of either the lactone or the acid. In further accordance with the findings of these two authors the acid showed mutarotation. An initial rotation of  $[\alpha]_D^{20} = +16$  and a maximum rotation of  $[\alpha]_D^{20} = +36$  obtained in 3 hours agree fairly well with the values obtained by Ehrlich and Rehorst, namely +12 and +36 respectively. At this point it might be interesting to recall that glycuronic acid monobenzoate (8) does not show mutarotation until it is neutralized. It is rather difficult to explain why the esterification of one of the hydroxyl groups should definitely hinder mutarotation. Glycuronic acid ought to be a type of compound which should lend itself well to further study of the relationship between optical rotation and the chemical structure of the carbohydrate molecule. In fact glycuronic acid with its two strongly positive groups should prove to be a useful substance for the study of carbohydrate chemistry in general, especially from a synthetic point of view, now that it can be prepared in large quantities.

#### EXPERIMENTAL.

*Preparation of Borneol Glycuronic Acid.*—5 gm. of pulverized borneol were fed daily to each of several dogs. The drug was incorporated directly with the food, or, which proved somewhat

more satisfactory, first suspended in a small volume of a gelatin-glucose mixture in order to insure better mixing with the food. The urine was collected, slightly acidified with acetic acid, and treated with lead acetate. The precipitate which carried down with it most of the coloring matter was filtered off, the clear light yellow filtrate heated to boiling, and an excess of zinc acetate added, whereupon a voluminous crystalline precipitate of zinc borneol glycuronate filled the container. The product was filtered off immediately and washed with hot water until no more coloring matter was extracted. In this way a pure white crystalline product of practically chemically pure zinc borneol glycuronate was obtained. The purity of this compound is emphasized because on it depends the success of preparing pure borneol glycuronic acid. The yield was about 1 gm. of the zinc salt for every gm. of borneol fed.

Glycuronic acid was prepared by dissolving the finely pulverized zinc salt in hot 3.5 N sulfuric acid, about 140 cc. of the acid for every 100 gm. of zinc borneol glycuronate being used. When the salt was completely dissolved, the solution was rapidly cooled and then placed in the ice box for several hours, whereupon the crystallization of the acid was complete. The crystalline mass was filtered off, washed with a small amount of cold water, and dried in the air. The product thus obtained was pure white and when redissolved gave a nearly colorless though somewhat turbid solution. The yield obtained was often as high as 87 gm. per 100 gm. of the zinc salt. On recrystallizing the product once from hot water and using a little decolorizing charcoal, one obtains a pure white material which when dissolved gave a water-clear solution.

*Analysis.*

Titration with 0.1 N sodium hydroxide.

Sample 0.5 gm. Required 14.25 cc. Calculated for  $C_{16}H_{24}O_7 \cdot 1H_2O$ , 14.4 cc.

Glycuronic acid.

Sample 0.1 gm. Found 0.0560 gm. Calculated for  $C_{16}H_{24}O_7 \cdot 1H_2O$ , 0.0558 gm.

*Preparation of Glycuronic Acid.*—100 gm. of borneol glycuronic acid were dissolved in 1500 cc. of 0.2 N, sulfuric acid, and the solution boiled for 3 hours under a reflux condenser. The borneol

was filtered off, and the hot filtrate treated with a sufficient amount of barium hydroxide to remove quantitatively the sulfuric acid. The complete removal of the mineral acid is essential since even traces of the acid will seriously interfere with the isolation of glycuronic acid. For the sake of convenience the barium sulfate was allowed to settle, the supernatant liquid siphoned off, and the final separation of the precipitate from the liquid effected by centrifuging. The colorless solution was concentrated under reduced pressure to a syrupy consistency. Standing caused crystallization to set in, and in the course of several hours the solution became almost a solid mass. The product was filtered off and washed with small amounts of alcohol until all traces of coloring matter were removed. The yield was 45 gm., or over 80 per cent of the theoretical.

*Analysis.*

Titration with 0.1 N sodium hydroxide.

Sample 0.2 gm. Titration to transitory end-point, 7.5 cc.

“ “ permanent “ 10.6 “

Composition of mixture calculated from titration: glycuronic acid 70.8 per cent, lactone 29.2 per cent.

Apparent molecular weight of mixture.

Calculated from total titration, 188.7.

“ on basis of 70.8 per cent acid and 29.2 per cent lactone, 188.8.

Glycuronic acid.

Sample 0.06 gm. Found 0.063 gm. Calculated on basis of 70.8 per cent acid and 29.2 per cent lactone, 0.062 gm.

*Separation of Glycuronic Acid from Its Lactone.*—The preparation of pure glycuronic acid from the mixture of acid and lactone, which is obtained by hydrolyzing borneol glycuronic acid, is most conveniently accomplished by extraction with alcohol. In this solvent the acid is less soluble than the lactone, and by extracting a sample several times practically all of the lactone is removed. The method entails no loss of material since the portion which is dissolved in the alcohol can easily be recovered. The exact procedure is best presented by citing an actual specific experiment.

4 gm. of the product containing 70.8 per cent acid and 29.2 per cent lactone were covered with 200 cc. of 95 per cent alcohol and set aside for 12 hours. The undissolved portion was filtered off and dried.

Yield 2.6 gm. Analysis: acid 77.5 per cent, lactone 22.5 per cent.

The remainder of the product (2.5 gm.) was again covered with 100 cc. of alcohol, and after 12 hours the product was again filtered off and dried.

Yield 2.2 gm. Analysis: acid 91 per cent, lactone 9 per cent.

Again the remaining portion was covered with 100 cc. of alcohol and allowed to stand for 24 hours. The temperature throughout the experiment was about 25°C.

Final yield 1.5 gm.

Titration with 0.1 N sodium hydroxide.

Sample 0.2 gm. Titration to transitory end-point, 10.15.

“ “ permanent “ 10.25.

Purity of glycuronic acid, 99 per cent.

*Optical Rotation.*—0.5 gm. of glycuronic acid was dissolved in 25 cc. of water. The initial rotation observed in 3 minutes after the substance was dissolved was  $[\alpha]_D^{20} = +16.5$ . The maximum rotation obtained in 3 hours and which remained constant for several days was  $[\alpha]_D^{20} = +36$ .

*Preparation of Lactone of Glycuronic Acid.*—5 gm. of the mixture of acid and lactone were dissolved in 25 cc. of hot glacial acetic acid. On cooling, the lactone alone crystallized out. The yield was 3 gm. of a slightly brown crystalline solid which when dissolved in water gave a solution which was neutral to litmus. A pure colorless preparation of the lactone was obtained by one recrystallization from hot water.

*Analysis.*

Titration with 0.1 N sodium hydroxide.

Sample 0.2 gm. Required 11.4 cc. Found 11.4 cc.

Glycuronic acid.

Sample 0.05 gm. Found 0.0558 gm. Calculated 0.0551 gm.

*Optical Rotation.*—0.5 gm. was dissolved in 25 cc. of water. The specific rotation observed was  $[\alpha]_D^{20} = +19$ .

#### SUMMARY.

Borneol glycuronic acid, because of the ease with which it can be obtained in large quantities, offers one of the best sources for the preparation of glycuronic acid. The procedure developed

for preparing borneol glycuronic acid consists in feeding borneol to dogs, isolating the conjugated glycuronic acid from the urine as the zinc salt, and finally preparing from this salt the free acid.

The hydrolysis of borneol glycuronic acid by means of dilute sulfuric acid yields a colorless crystalline product containing approximately 70 per cent of glycuronic acid and 30 per cent of the lactone. From this mixture pure glycuronic acid can be prepared by extraction with alcohol since the lactone is more soluble in that solvent than the acid. A convenient method for preparing the lactone consists in dissolving the mixture in hot glacial acetic acid from which the lactone alone crystallizes on cooling. The properties of the acid and the lactone correspond to those described in the literature.

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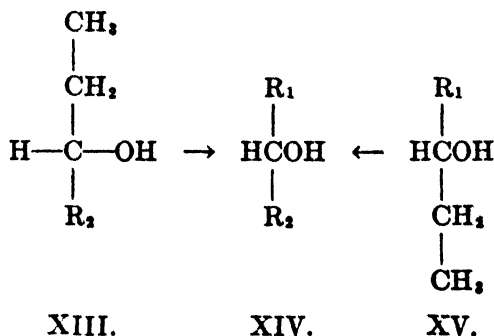
# CONFIGURATIONAL RELATIONSHIPS OF 2-HYDROXY-BUTYRIC AND LACTIC ACIDS.

By P. A. LEVENE AND H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research  
New York.)

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The correlation of the configurations of the individual  $\alpha$ -hydroxy acids has not yet been established by direct chemical methods. Indirect methods were suggested by Clough, by Hudson, and by Levene. It is therefore important to test by the direct method the conclusions reached by the indirect methods. The plan for the direct chemical method is the following. Dextro-lactic acid was shown to be configurationally related to dextro-3-hydroxybutyric acid ( $\beta$ -hydroxybutyric acid) and to dextro-1,3-dihydroxybutane and the latter in its turn has been correlated with dextro-methylethyl carbinol. From the figures on p. 344 it is seen clearly that 2-hydroxybutyric acid having the hydroxyl on the same side as dextro-3-hydroxybutyric acid should lead to a levo-methylethyl carbinol; whereas 2-hydroxybutyric acid, configurationally related to levo-3-hydroxybutyric acid, should lead to dextro-ethylmethyl carbinol. To make the reasoning more comprehensible, one may represent the relationship of dextro- and levo-ethylmethyl carbinols to dextro-lactic acid in the following way.



It is evident that when  $\text{R}_1$  (in XV) is made to be the same group as  $\text{R}_2$  (in XIII) then (XIII) will be enantiomorphous to (XV).





The plan of the work leading to the correlation of the configuration of 2-hydroxybutyric acid with lactic acid and with methylethyl carbinol was analogous to that which led to the correlation of 3-hydroxybutyric acid with lactic acid and with methylethyl carbinol.

The transformation of dextro-2-hydroxybutyric acid into levo-1,2-dihydroxybutane may be regarded as the first step in the work. The second step consisted of the preparation of 1,2-dihydroxybutane starting from chloromethylethyl ketone, the conversion of this compound into hydroxymethylethyl ketone, and the reduction of the latter by fermentation to dextro-1,2-dihydroxybutane. The glycol was converted into the levo-chlorohydrin, which was finally reduced to dextro-ethylmethyl carbinol. Hence, levo-1,2-dihydroxybutane derived from dextro-2-hydroxybutyric acid will yield levo-ethylmethyl carbinol. This result, as pointed out above, is to be expected if dextro-2-hydroxybutyric acid has the same configuration as dextro-lactic acid; namely, if it belongs to the *l* series of hydroxy acids.

The opposite conclusion reached by Clough was based on an error, inasmuch as the direction of the rotation of the free acid was not taken into consideration by this author. Clough designated as levo-hydroxybutyric acid that acid the salt of which was levorotatory. From the data presented in this paper, it is seen that the levorotatory ammonium salt of 2-hydroxybutyric acid leads to a dextrorotatory free acid.

On the other hand, the rule of Levene for determining the configuration of 2-hydroxy acids on the basis of the sign of the difference of the rotation of the undissociated acid and of its ion is fully substantiated by the method developed in the present communication.

#### EXPERIMENTAL.

*Levo- and Dextro-2-Hydroxybutyric Acids.*—The inactive acid was obtained from 2-bromobutyric acid by the procedure described by Bischoff and Walden.<sup>1</sup>

An aqueous solution of the free acid was neutralized with morphine; the solution was heated on the steam bath for half an hour and then filtered. The filtrate was concentrated under reduced

<sup>1</sup> Bischoff, C. A., and Walden, P., *Ann. Chem.*, 1894, cclxxix, 102.

pressure to a thick syrup. When this was left in the ice box overnight, part of it crystallized. The crystals were removed on a Buchner funnel and then twice recrystallized from 50 per cent alcohol.

The morphine salt was dissolved in water and decomposed by the addition of a slight excess of ammonia. The morphine was removed by filtration and the filtrate converted into the barium salt in the usual way. In water the barium salt had the following rotation.

$$[\alpha]_D^{25} = \frac{+ 0.43^\circ \times 100}{1 \times 5.6} = + 7.7^\circ.$$

A barium salt prepared from the acid obtained on decomposition of the mother liquors in the above resolution yielded the following rotation.

$$[\alpha]_D^{22} = \frac{- 0.56^\circ \times 100}{2 \times 5.0} = - 5.6^\circ.$$

The free acid was liberated from the second salt in the following manner. 2.0 gm. of barium salt ( $[\alpha]_D^{22} = -5.6^\circ$ ) were dissolved in cold water and 5.0 cc. of 2.32 N hydrochloric acid added. The volume was made up to 10 cc. and the rotation taken immediately.

$$[\alpha]_D^{22} = \frac{+ 0.54^\circ \times 100}{2 \times 12} = + 2.3^\circ.$$

*Ethyl-Levo-n-2-Hydroxybutyrate*.—100 gm. of thoroughly dried barium 2-hydroxybutyrate ( $[\alpha]_D^{22} = -5.6^\circ$ ) were suspended in 250 cc. of absolute alcohol and a solution of 34 gm. of concentrated sulfuric acid in 100 cc. of absolute alcohol was slowly dropped in, the mixture being vigorously stirred with a mechanical stirrer. The mixture was heated under a reflux condenser on a boiling water bath for 8 hours. After cooling, dry ether was added, the excess sulfuric acid was neutralized with solid potassium carbonate, and the solution filtered from salts. It was then dried over anhydrous sodium sulfate, the solvent removed, and the ester was distilled under reduced pressure. It boiled at 64–66°C.,  $p = 20$  mm., and rotated without solvent as follows:

$$[\alpha]_D^{25} = \frac{- 3.75^\circ \times 1}{1 \times 0.978} = - 3.83^\circ.$$

It analyzed as follows:

0.1134 gm. substance: 0.2264 gm. CO<sub>2</sub> and 0.0938 gm. H<sub>2</sub>O.

C<sub>6</sub>H<sub>12</sub>O<sub>4</sub>. Calculated. C 54.40, H 9.16.

Found. " 54.44, " 9.25.

An ester prepared from a barium salt ( $[\alpha]_D^{24} = +7.7^\circ$ ) had the following rotation.

$$[\alpha]_D^{22} = \frac{+8.40^\circ \times 1}{1 \times 0.978} = +8.59^\circ.$$

*Levo-1,2-Dihydroxybutane*.—Ethyl-2-hydroxybutyrate ( $[\alpha]_D^{22} = -3.8^\circ$ ) was reduced with sodium and glacial acetic acid in the apparatus described by Levene and Allen.<sup>2</sup> The procedure was the same as that described previously for the reduction of other hydroxy acids. The glycol boiled at 94–96°C., p = 12 mm.

It analyzed as follows:

0.0936 gm. substance: 0.1827 gm. CO<sub>2</sub> and 0.0916 gm. H<sub>2</sub>O.

C<sub>4</sub>H<sub>10</sub>O<sub>2</sub>. Calculated. C 53.27, H 11.20.

Found. " 53.22, " 10.95.

27 gm. of ethyl-2-hydroxybutyrate yielded 2 gm. of the glycol.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{-0.63^\circ \times 100}{2 \times 4.25} = -7.4^\circ.$$

*Di-(Phenylurethane) of Levo-1,2-Dihydroxybutane*.—The urethane was prepared in the usual way. Recrystallized from dilute alcohol several times, it melted at 121–123°C. and analyzed as follows:

0.1000 gm. substance: 6.33 cc. 1 N HCl.

C<sub>18</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub>. Calculated. N 8.54.

Found. " 8.86.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{-2.28^\circ \times 100}{2 \times 5.74} = -19.8^\circ.$$

<sup>2</sup> Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 443.

*Preparation of Optically Active 1,2-Dihydroxybutane from Methyleneethyl Ketone.*

*Chloromethyleneethyl Ketone (1-Chlorobutanone-2).*—This product was obtained by the chlorination of methyleneethyl ketone, the procedure employed being essentially the same as that described by Kling.<sup>3</sup> The variation introduced was that twice as much marble was used. After the reaction mixture had been dried over calcium chloride it was distilled under reduced pressure. It was then subjected to repeated fractional distillations with an efficient fractionating column. A fraction which boiled constantly at 138.8–139.2°C. (corrected),  $p = 755$  mm., was obtained.

It analyzed as follows:

0.1228 gm. substance: 0.1694 gm. AgCl.

$C_4H_7OCl$ . Calculated. Cl 33.33.

Found. " 34.12.

*Hydroxymethyleneethyl Ketone (Butanol-(1)-one-(2)).*—100 gm. of chloromethyleneethyl ketone (b.p. = 138.8–139.2°C.), 160 gm. of dried potassium formate, and 160 cc. of dry methyl alcohol were heated under a reflux condenser on a water bath overnight.

The reaction mixture was cooled, dry ether was added, and the solution was then filtered. The solvent was removed and the hydroxy ketone distilled under reduced pressure. It was then redistilled under reduced pressure employing a flask provided with a Vigreux column. The fraction, which boiled at 50.5–51°C.,  $p = 14$  mm., was collected and used for reduction to the glycol.

*Dextro-1,2-Dihydroxybutane.*—This glycol was obtained by the reduction of hydroxymethyleneethyl ketone with fermenting yeast.

To an actively fermenting mixture of 45 gm. of cane sugar, 450 gm. of bakers' yeast, and 4500 cc. of water were added 45 gm. of hydroxymethyleneethyl ketone. The reaction mixture was allowed to stand 6 days and then worked up in the usual way.<sup>4</sup> The glycol thus obtained on redistillation from a flask provided with a Vigreux column boiled at 75.0–75.5°C.,  $p = 1$  to 1.5 mm. Another lot distilled at 91–91.5°C.,  $p = 13$  mm.

<sup>3</sup> Kling, A., *Compt. rend. Acad.*, 1905, cxi, 312.

<sup>4</sup> Neuberg, C., and Kerb, E., *Biochem. Z.*, 1918, xcii, 96. Farber, E., Nord, F. F., and Neuberg, C., *Biochem. Z.*, 1920, cxii, 313.

It analyzed as follows:

0.1076 gm. substance: 0.2104 gm. CO<sub>2</sub> and 0.1050 gm. H<sub>2</sub>O.

0.1238 " " 0.2397 " " " 0.1252 " "

C<sub>4</sub>H<sub>10</sub>O<sub>2</sub>. Calculated. C 53.27, H 11.20.

Found. " 53.32, " 10.92.

" 52.79, " 11.31.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{+ 2.00^\circ \times 100}{1 \times 16.1} = + 12.4^\circ.$$

*Di-(Phenylurethane) of Dextro-1,2-Dihydroxybutane.*—This substance was prepared in the usual way. Several recrystallizations from dilute alcohol gave a product which melted at 125–127°C. and analyzed as follows:

0.1000 gm. substance: 5.90 cc. 1 N HCl.

C<sub>18</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub>. Calculated. N 8.54.

Found. " 8.26.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{+ 1.08^\circ \times 100}{1 \times 4.55} = + 23.7^\circ.$$

### *Conversion of Optically Active 1,2-Dihydroxybutane into Secondary Butyl Alcohol.*

*Levo-1-Bromo-2-Hydroxybutane.*—This bromohydrin was prepared from dextro-1,2-dihydroxybutane ( $[\alpha]_D^{22} = +12.4^\circ$ ) in the usual way.<sup>5</sup>

It boiled at 61–63°C., p = 12 mm. The optical rotation in a 1 dm. tube without solvent was  $\alpha_D^{22} = -11.8^\circ$ , t = 22°C.

*Dextro-Ethylmethyl Carbinol.*—The bromohydrin obtained in the above experiment was reduced in alkaline solution with hydrogen in the presence of colloidal palladium by the procedure previously described.<sup>5</sup> After drying over anhydrous sodium sulfate, the ether was removed with the aid of a Vigreux column. The alcohol

<sup>5</sup> Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxi, 465. Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 591.

## 350      2-Hydroxybutyric and Lactic Acids

was then distilled and a fraction which boiled at 98–99°C. was collected. In alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{+ 1.15^\circ \times 100}{1 \times 8.82} = + 13.0^\circ.$$

It was converted into the phenylurethane in the usual manner. This analyzed as follows:

0.1000 gm. substance: 5.00 cc. 1 N HCl.

C11H15O2N. Calculated. N 7.26.

Found.        " 7.00.

It melted at 61–63°C. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{+ 1.08^\circ \times 100}{1 \times 4.08} = + 26.5^\circ.$$

# THE CONDUCTIVITY METHOD AND PROTEOLYSIS.

## I. EXPERIMENTS ON PEPTONE.\*

By HARRY D. BAERNSTEIN.

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.*)

(Received for publication, May 25, 1927.)

### INTRODUCTION.

In earlier studies from this laboratory the problem of initial cleavage of proteins as contrasted with secondary cleavage was attacked. The methods used were not found to be wholly adequate for the solution of these problems and so a search for other methods was begun.<sup>1</sup> From the work of Northrop and others on the conductivity method, it seemed worth while to study this technique with a view to employing it in our studies of proteolysis. The purpose of this communication is to characterize the conductivity changes occurring in a peptic digest further than has been done by Northrop and others who have employed this technique. The problem arose from an attempt to measure the rate of digestion at various pH levels. We have found that above a pH of 3 the conductivity always increased in peptic digestion, whereas below this level the conductivity decreased. There was a maximum rate of decrease at about pH 1.3, which we believed did not represent the optimum pH for peptic digestion so much as it represented the optimum pH for the binding power of the split-products. In order to study this phenomenon, various prepara-

\* This paper was submitted in partial requirement for the degree of Doctor of Philosophy.

The writer wishes to express his gratitude to Dr. H. C. Bradley of this Department for his aid in carrying out this work, and in preparing this manuscript.

<sup>1</sup> The references from 1 to 20 in the bibliography represent important contributions to the problem of measuring proteolysis.



tions containing the products of protein cleavage were added to solutions of HCl of different pH, and the effect on the conductivity determined.

### *Apparatus.*

The apparatus used consisted of a water thermostat controlled by a mercury-toluene regulator (21). The temperature was  $37^{\circ}\text{C.} \pm 0.1^{\circ}\text{C.}$  in all the experiments. A Leeds and Northrup Wheatstone bridge was employed for measuring resistances. It was found convenient to attach an extension arm of hard rubber,

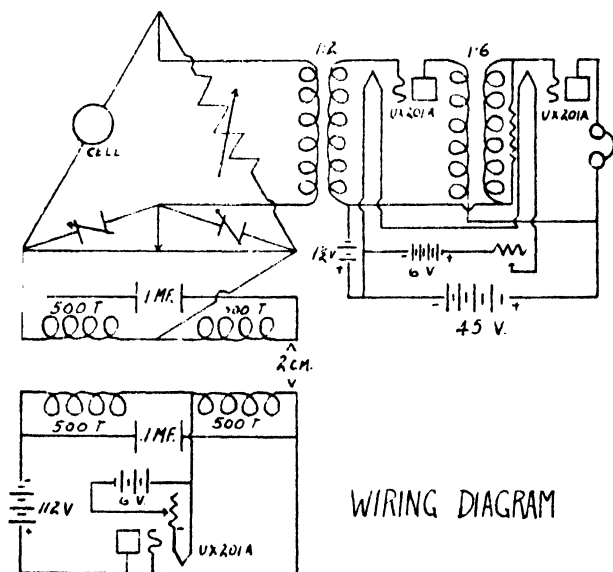


FIG. 1.

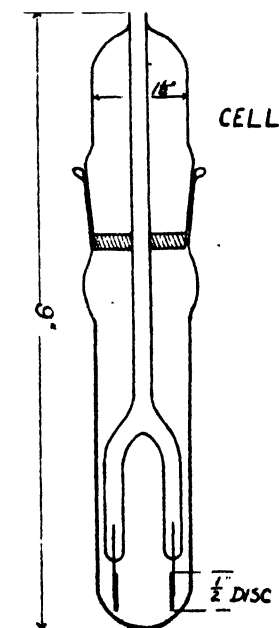


FIG. 2.

about a foot long, to the fine adjustment dial in order to eliminate changes in capacitance of the circuit due to close proximity of the hand when making adjustments. The source of oscillating current was a radio tube with appropriate coils and condensers modified from the Hall and Adams arrangement (22). The frequency was constant at 5120 cycles. A two stage radio tube amplifier was introduced into the telephone circuit to increase the sensitivity. Fig. 1 shows the wiring diagram of the set up.

The conductivity cells were made by the University glass

blower from the sort of ground glass joints used in high vacuum apparatus (Fig. 2). The hydrogen ion measurements were made by the electrometric method.

### *Materials.*

The egg albumin for the substrates was prepared from fresh eggs as follows: 4 dozen egg whites were thoroughly mixed and passed through several layers of gauze. Total nitrogen was determined on 5 cc. samples and found to be 0.0171 gm. per cc. Total nitrogen was determined on Merck's powdered egg albumin and found to be 11.39 per cent N. A 3 per cent egg albumin solution contains  $0.03 \times 11.39 = 0.3417$  gm. of N per 100 cc. The number of cc. of egg white necessary to make a 3 per cent solution is then  $\frac{0.3417}{0.0171}$  or 20 cc. of egg white per 100 cc. of solution.

Accordingly 20 cc. of egg white were mixed with various quantities of normal HCl and diluted to 100 cc. to make our stock substrate solutions. (No acid was added to solution of pH 9.26.)

Witte's peptone was dissolved in water, filtered, and evaporated to a scale and powdered. This procedure was necessary in order to hasten the process of solution in the experiments. Seibert (23) found that this peptone contained 47.63 per cent of its nitrogen as protein, 44.55 per cent as proteose, and 7.81 per cent as residual nitrogen. It therefore represents the first cleavage products of native protein. Davis (24) found the following for Witte's peptone: total N 14.52 per cent, moisture 6.33 per cent, ash 3.44 per cent.

The amino acids were obtained from the Special Chemicals Company, Waukegan, Illinois.

The Parke, Davis and Company peptone is the same as is used in our bacteriological laboratory for culture media. It gives no precipitate with trichloroacetic acid of 2 per cent final concentration, but is almost completely precipitated by saturated  $\text{Na}_2\text{SO}_4$  at 33°C. It is therefore largely proteose and peptone.

The preparation called aminoids (Arlington Chemical Company, Yonkers) was obtained on the market, and represents dipeptides and amino acids. It does not give a biuret reaction.

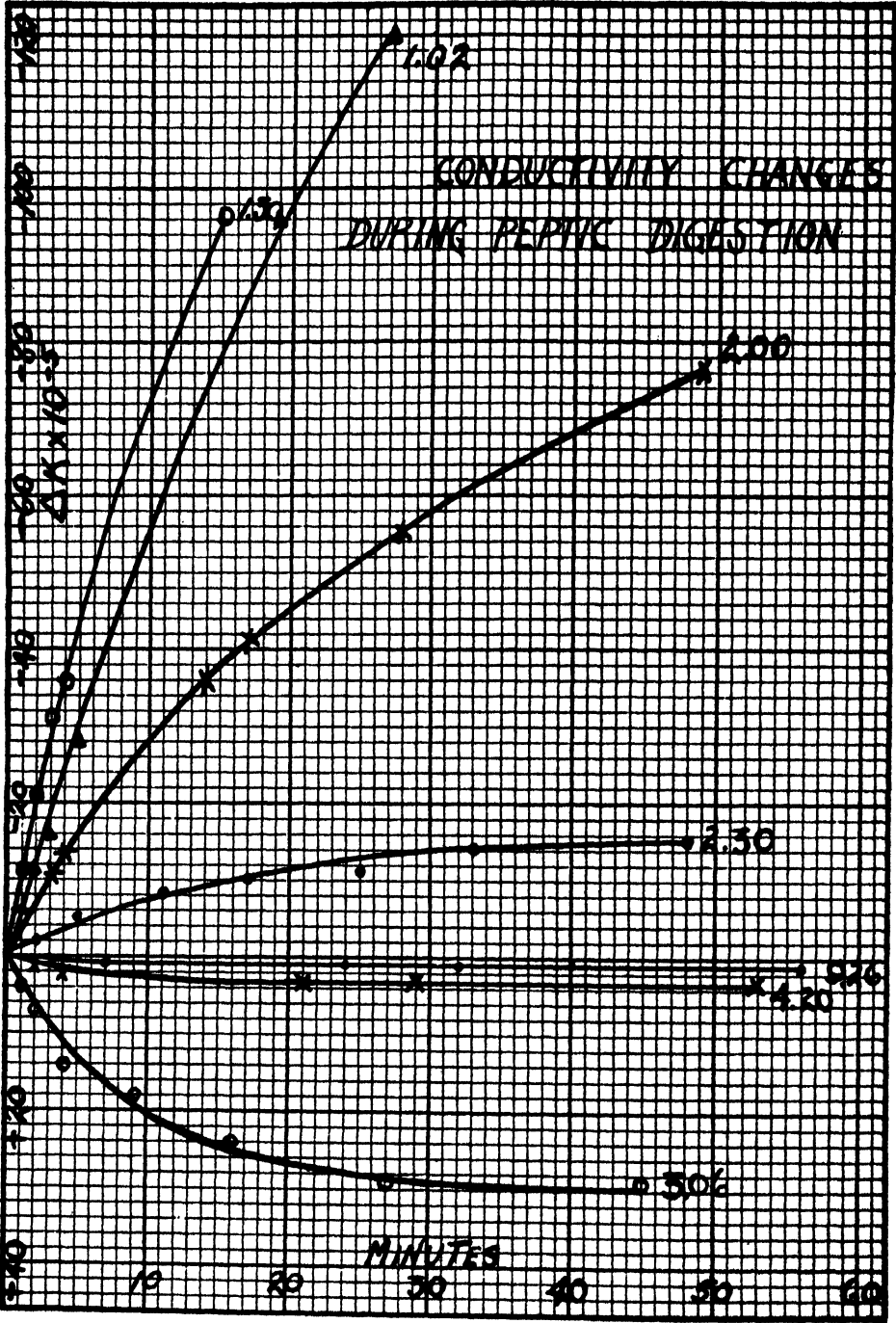


Fig. 3. The figures at the end of the curves denote pH of solution.

*Results.**Experiment I. Changes in Conductivity during Peptic Digestion at Various pH Levels.*

The technique consisted in measuring 25 cc. of each of the standard substrate solutions into the conductivity cells and, after allowing for thermal equilibrium, measuring the resistance. A 4 per cent solution of U.S.P. 1:3000 pepsin was made up and allowed to reach thermal equilibrium. 1 cc. of this was then quickly introduced into the first cell, and the stop-watch started. The mixture was shaken in the thermostat for exactly 1 minute, and then at the end of the 2nd minute the resistance was recorded. The resistance was recorded at the end of the 3rd minute, and then the second cell started in the same way. This was repeated for the third and fourth cells. A reading was taken at intervals on all cells for a period of about 1 hour. The conductivities were then calculated and plotted on a large scale. The conductivity at zero time was found by extrapolation. All other conductivities were then subtracted from this figure to obtain  $\Delta K$  values. If  $\Delta K$  is negative the conductivity decreases, and if positive the conductivity increases. These values were then plotted against the time, and smooth curves were drawn through the points. Fig. 3 shows the result of this experiment. It will be observed that the  $\Delta K$  changes from positive to negative somewhere between pH 3.06 and 2.3. There is maximum rate of change of conductivity between pH 1.30 and 1.02. The curves are not straight lines, which indicates a falling off in the rate of change in conductivity. What is the cause of this phenomenon? Northrop (25) showed that the destruction of pepsin is not adequate to account for the decreased rate of reaction. He found, however, that pepsin is bound by the digestion products. This may account for some of the change, although it is not likely since the falling off in rate is more striking at pH levels where pepsin is least active in producing split-products. It has been abundantly demonstrated that the products bind HCl. This decrease in concentration of HCl could effect the changes through several mechanisms. First, the enzyme certainly becomes less active; second, the equilibrium between protein and acid protein salt may be disturbed, reducing the available substrate (5); third, the ability of peptone to change

the conductivity may be less at the higher pH. It is this last factor which we have endeavored to establish in Experiment II. We believe that the change in sign of  $\Delta K$  is caused by the change in the ionization of the amphoteric split-products, and we shall present some data substantiating this in Experiment III.

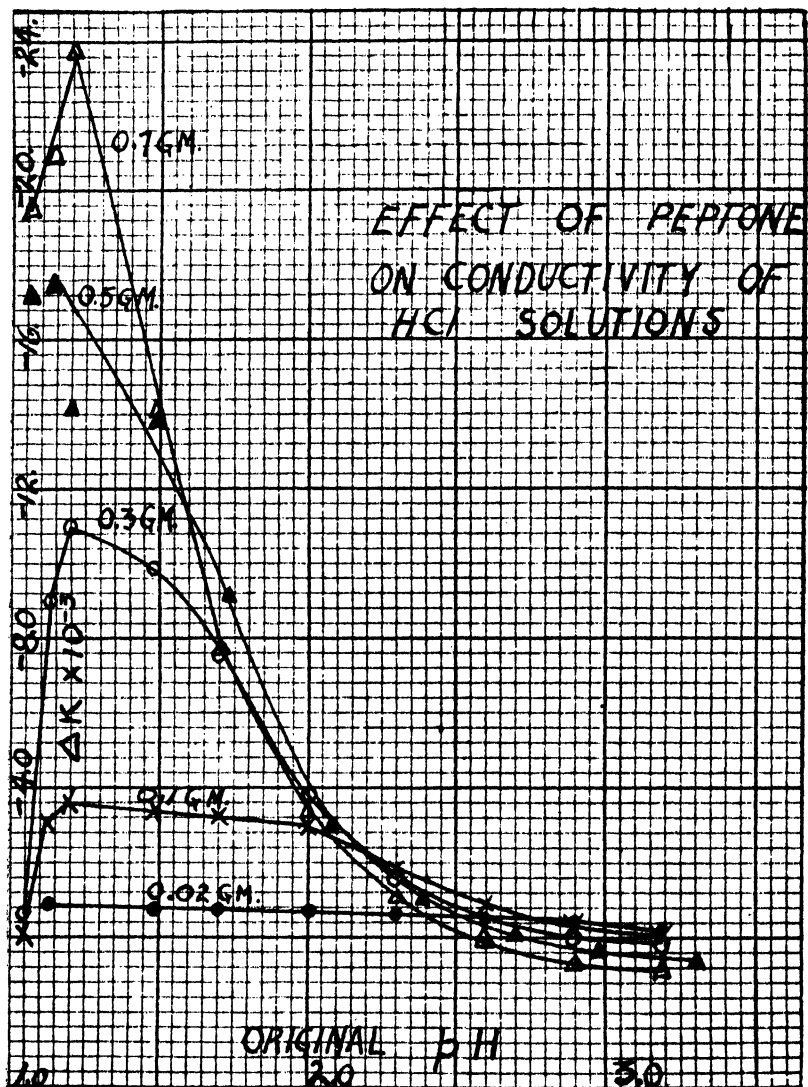


FIG. 4.

The maximum rate of change at about pH 1.3 is probably in part due to the activity of the enzyme, but we believe it is largely due to the relation between the acid content of the solution and

the binding power of the products. The next experiment furnishes some data on this point also.

*Experiment II. Effect of Witte's Peptone on Conductivity of HCl Solutions.*

The technique consisted in measuring 25 cc. of HCl solutions of various pH values into the cells and measuring their resistances at 37°C. 0.02 gm. of peptone was then introduced into each of the cells and allowed to dissolve. The resistance was again measured and the procedure repeated with 0.08, 0.2, 0.4, and 0.6 gm. The conductivities were then obtained and subtracted from the conductivity of the HCl solution; this gave the  $\Delta K$  values. These values were then plotted against the original pH of the solution, and the curves of Fig. 4 drawn.

It will be observed that small amounts of peptone decrease the conductivity over the range pH 3.2 to 1.0. Larger amounts increase the conductivity above pH 3 and decrease it below pH 3. There is a maximum effect at pH 1.2.

We have therefore established two points: first, that Witte's peptone has a variable effect on the conductivity which depends on the pH of the solution; second, that the change in sign of  $\Delta K$  at pH 3, and the maximum at pH 1.2, occurring in a peptic digestion may be reproduced by the simple addition of peptone to acid solutions. We shall discuss these questions further after we have examined the causes of the phenomena.

*Experiment III. Effect of Various Cleavage Products on Conductivity at Various pH Levels.*

For this purpose we endeavored to obtain preparations representing the stages of degradation of the protein molecule. Witte's peptone contains the largest fragments, Parke, Davis and Company peptone the medium sized ones, aminoids the smaller peptides, and amino acids the smallest fragments. The technique was the same as that employed in Experiment II except that 0.1 gm. of material was used throughout. The results appear graphically in Fig. 5.

The peptides had the greatest effect in increasing the con-

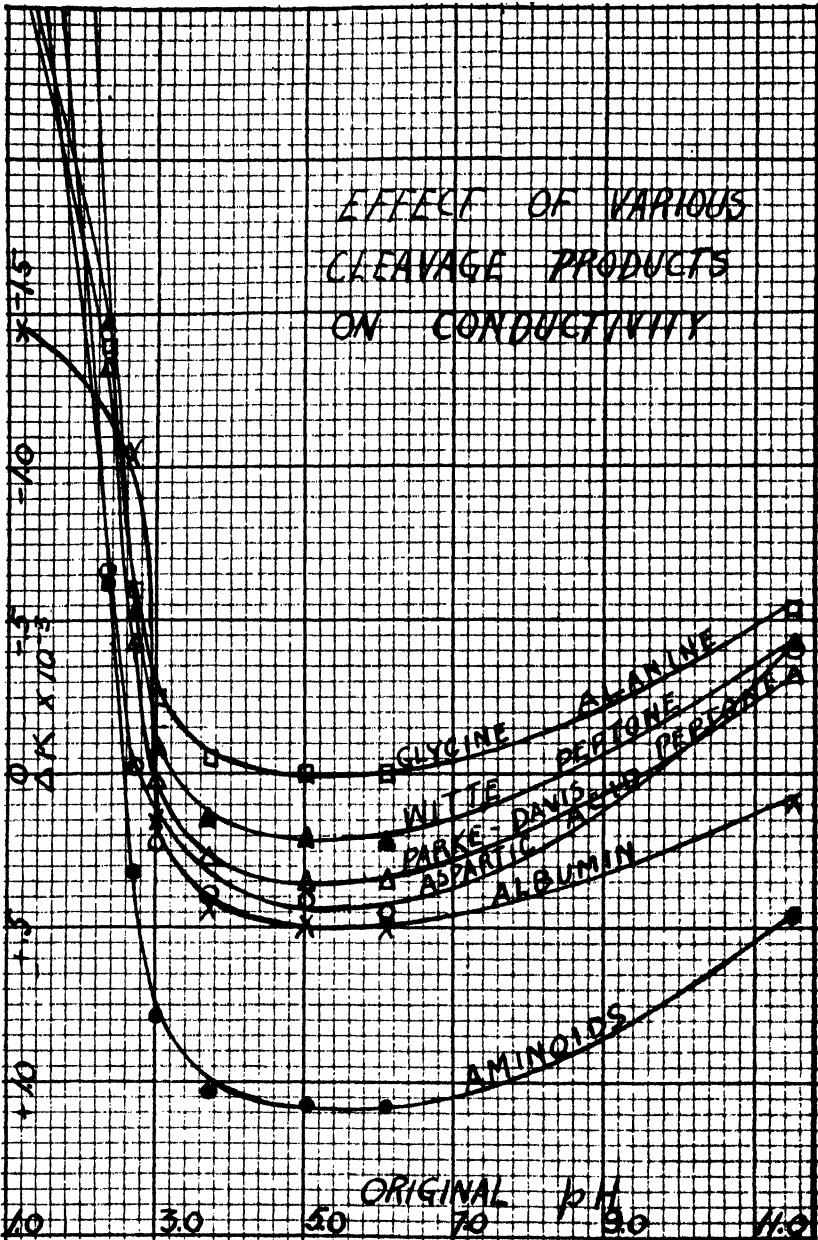


FIG. 5.

ductivity, and the amino acids decreased the conductivity most. The proteoses showed a medium effect in both directions, while the meta-protein had the least effect.

## DISCUSSION.

In order to clarify the situation, we have listed the products according to their effect in increasing the conductivity at pH 6.1.

Opposite each value we have given the number of cc. of 0.1 N NaOH required to neutralize 10 cc. of a 2 per cent solution of each material to phenolphthalein (Table I).

TABLE I.  
*Comparison of  $\Delta K$  at pH 6.1 with Titration.*

Solution.	0.1 N NaOH.
	cc.
Aminoids.....	2.2
Albumin.....	0.3
Aspartic acid.....	
Parke, Davis and Company peptone.....	1.80
Witte peptone.....	0.78
Glycine.....	
Alanine.....	0.40

TABLE II.  
*Comparison of  $\Delta K$  at pH 1.17 with Titration after Formaldehyde.*

Solution.	$\Delta K \times 10^{-3}$	0.1 N NaOH.
		cc.
Glycine.....	15.44	
Alanine.....	13.58	10.85
Aspartic acid.....	8.37	
Aminoids.....	7.88	7.37
Parke, Davis and Company peptone. ....	4.77	2.75
Witte peptone.....	3.88	1.37
Albumin.....	1.45	0.67

The following generalization seems justified. The greater the effect of these substances on the conductivity at pH 6.1, the more alkali is required to neutralize them. In the case of albumin the effect on the conductivity is more than we should expect. This we believe is due to salt impurities contained in the preparation. The insoluble residue in this case and with Witte's peptone appeared to be meta-protein. We believe, on the basis of this experi-



ment, that the increase in conductivity is due to the ionization of the substances as acids according to the equation:



Part of this acid is of course used to neutralize the amino groups present.

We have next arranged the substances in the order of their ability to decrease the conductivity at pH 1.17. Opposite each value we have given the number of cc. of NaOH required to neutralize the above neutral samples after the addition of 5 cc. of formaldehyde. This procedure measures the amino nitrogen which was masked by the acids present (Table II).

We therefore believe that the ability to decrease the conductivity is dependent on the amount of amino nitrogen contained in the preparations. It is also dependent on the amount of HCl available (Experiment II). By applying the mass law to these phenomena, we can get a satisfactory explanation of the mechanisms concerned.

Let  $\text{RNH}_2\text{COOH}$  represent the products of hydrolysis. Then



These substances are all in equilibrium. Increasing the concentration of the first member results in a diminution of the concentration of HCl and a further decreasing of the conductivity. This was observed. Increasing the concentration of HCl reduces the concentration of the first member. If this proceeds to completion, then further addition of acid tends to increase the conductivity. This accounts for the maximum at pH 1.2.

We have now sufficient data, we believe, to interpret the curves obtained in Experiment I. At pH 9.26 pepsin was inactive and did not produce any decomposition products. The very slight increase in conductivity was probably due to temperature changes produced by the introduction of the pepsin. At pH 4.20 there was an initial slight cleavage producing some acid groups, and thus increasing the conductivity. Cleavage probably did not continue at a measurable rate due to the low activity of pepsin at this pH and its removal by the products. At pH 3.06 cleavage continued for a longer time, since pepsin can act fairly well at this

acidity. The reduced rate is probably due to the removal of pepsin by the products. At 2.30 pepsin was more active, but the products bound HCl, thus causing a reversal of sign of  $\Delta K$ . The reduced rate here was due to two factors: first, the removal of pepsin, second, the removal of HCl by the products, thus increasing the pH and preventing the substances formed from exercising their full effect on the conductivity as shown by application of the mass law. At pH 2.00 the decrease in rate was not so marked due to the increased amount of acid available. The decrease was still less marked at pH 1.30. At pH 1.02 the rate of change of conductivity was actually less than at pH 1.30 because there was an excess of HCl present over that necessary to unite with all of the products.

#### SUMMARY.

1. The differences noted in the rate of digestion of egg albumin with pepsin at various pH levels as measured by the conductivity method are largely due to the variable effect of the products of digestion on the conductivity.
2. An explanation is advanced to account for the increases in conductivity above pH 3.0 and the decreases below pH 3.0.
3. The ability of the products to increase the conductivity runs parallel to their free acid content and their ability to decrease the conductivity runs parallel to their amino nitrogen content.
4. The falling off in rate of change in conductivity is due to the equilibrium conditions existing between the products and the acid present.
5. This method is not the one of choice for determination of the optimum hydrogen ion concentration for peptic activity because of the variable effect of the products of digestion on the conductivity at various hydrogen ion levels.

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## DETERMINATION OF LACTIC ACID IN BLOOD.

By ETHEL RONZONI AND ZONJA WALLEN-LAWRENCE.

*(From the Department of Internal Medicine, Washington University  
School of Medicine, St. Louis.)*

(Received for publication, June 2, 1927.)

Preparatory to a study of the glycolytic activity of blood and other tissues, the following analysis of the accuracy of analytical methods for the determination of lactic acid was made. Blood was used throughout these experiments because of the ease with which it can be handled, and we may assume that, with slight modifications, the procedure which will be described might be applied to other tissues.

The methods used for the determination of lactic acid are those commonly employed. The results obtained by permanganate oxidation method as modified by Friedemann, Cotonio, and Shaffer (3), collecting the aldehyde thus yielded in bisulfite and titrating as described by Clausen (1), are compared with those obtained by the sulfuric acid dehydration method, Clausen's procedure being used throughout except that the reaction temperature is kept between 160–170°C. instead of at 140° as originally recommended by him. Both aldehyde and carbon monoxide liberated by this method have been determined. The carbon monoxide is freed from bisulfite and water by passing through sodium hydroxide and concentrated sulfuric acid and is determined by the iodine pentoxide method (5).

Friedemann, Cotonio, and Shaffer are able to recover  $98 \pm 3$  per cent of lactic acid from pure solutions as aldehyde, using the permanganate oxidation method. Clausen has shown a similar yield and consistency in results using the sulfuric acid method. Applying the procedures outlined by these workers, we find our accuracy of the same order as theirs when analyzing solutions containing small amounts of lactic acid, 0.2 to 2 mg. If the lactic acid is calculated from the carbon monoxide formed in the sulfuric

acid procedure, the determination is quantitative within the limits of error of the iodine titration.

Varying the volume of solution in the reaction flask does not affect the recovery of aldehyde. 50 cc. of normal sulfuric acid containing 0.01 molar manganous sulfate may be diluted with from 5 to 15 cc. of solution containing lactic acid without influence on the determination, and variations in the amount of sulfuric acid from 35 to 80 cc. do not affect the results.

In connection with the determination of lactic acid in pure solution the effect of standing in solution should be noted. During the first 3 days, a solution of zinc lactate shows a drop in the aldehyde yield from day to day until on the 3rd day, it reaches a constant level (Table I), yielding about 84 per cent of the theoreti-

TABLE I.  
*Effect of Standing on Amount of Lactic Acid Recovered from Zinc Lactate Solution.*

Solution I. Room temperature 32°C.			Solution II. Room temperature 26°C.		
Age.	No. of analyses.	Per cent of original.	Age.	No. of analyses.	Per cent of original.
<i>days</i>			<i>days</i>		
Fresh.	7	97.2	Fresh.	6	98.2
1	4	93.41	1	6	94.6
2	4	86.24	2	6	90.2
3	4	84.07	3	6	86.2
5	6	84.29	5	6	84.9
10	4	84.2	14	6	84.6
21	4	84.4	21	6	84.7

cal amount. A state of equilibrium is evidently reached at which it remains constant for as long as 3 weeks. We are unable at the present time to explain the cause for this disappearance of lactic acid. Dakin's (2) early observations on the formation of methylglyoxal in lactic acid solutions on standing lead to speculation along the same line in cases of zinc lactate solutions. The loss is presumably not bacterial, since it occurs in sterile solutions and in acid solutions containing mercuric chloride.

After testing the procedures on pure solutions, we took up the study of the methods for separation of lactic acid from protein and other substances preliminary to its determination. The possible

interference of other substances is dealt with in this paper only in so far as they may or may not be removed by the methods of protein precipitation employed.

TABLE II.

*Comparison of Permanganate and Sulfuric Acid Methods on Tungstic Acid Blood Filtrates.*

10 cc. samples of 1:10 dilution were used for determination by permanganate and 5 cc. used by sulfuric acid. Results expressed as mg. per 100 cc. of blood.

Blood.	KMnO <sub>4</sub>	H <sub>2</sub> SO <sub>4</sub>	Increase by H <sub>2</sub> SO <sub>4</sub> .	Per cent increase by H <sub>2</sub> SO <sub>4</sub> .
Beef blood and Zn lactate.....	227.9	256.5	28.6	11.1
Human blood.....	38.5	43.6	5.1	13.2
“ “.....	24.2	29.8	5.6	23.1
Beef “ old.....	71.5	80.6	9.1	12.7
“ “ and Zn lactate.....	134.5	119.7	15.2	11.3
Human blood of same individual during sugar tolerance.....	22.6	33.5	10.9	48.4
	29.5	46.5	17.0	57.6
	24.0	35.2	11.2	46.6
	21.4	33.9	12.5	58.4
Blood before exercise.....	18.2	28.3	10.1	55.4
Same subject after exercise.....	108.0	118.6	10.6	9.8
High non-protein N 140 mg.....	16.3	24.4	8.1	49.7
“ “ “ “ 112 “.....	18.3	28.4	10.1	55.2
Blood after exercise.....	101.7	114.7	13.0	12.7
Same individual during sugar tolerance.	19.0	32.0	13.0	68.4
	24.0	39.3	15.3	63.8
	19.1	32.0	12.9	68.5
	24.4	35.4	11.0	45.2

*Comparison of the Permanganate and Sulfuric Acid Methods on Blood Filtrates.*

With tungstic acid filtrates, we regularly obtain higher values by sulfuric acid dehydration than by the permanganate oxidation. This is contrary to the findings of Clausen who reports higher yields by the permanganate method. The fact that we use a higher reaction temperature for the sulfuric acid reaction may in part

account for this discrepancy. As shown in Table II, the differences between the two methods show an average value of 10.2 mg., calculated per 100 cc. of blood. The amount bears no direct relationship to the amount of lactic acid being determined.

Since carbon monoxide is also liberated by the sulfuric acid reaction ( $\text{CH}_3\text{CHOH}\cdot\text{COOH} = \text{CH}_3\text{COH} + \text{CO} + \text{H}_2\text{O}$ ), it is of interest to compare the results from the determination of carbon monoxide with the aldehyde titration by the sulfuric acid and permanganate methods. Table III gives the results of several such experiments and makes it clear that the lactic acid calculated from the carbon monoxide checks with that calculated from the bisulfite bound when the permanganate method is used. It seems probable,

TABLE III.  
*Comparison of Lactic Acid Recovery by Three Methods.*

Results expressed as mg. per 100 cc. of blood.

No. of determinations.	$\text{H}_2\text{SO}_4$		$\text{KMnO}_4$	
	Aldehyde titration.	CO	Aldehyde titration.	
2	80.6	70.9	71.7	Old blood.
4	56.3	46.3	47.2	“ “
2	75.0	63.2	63.3	Blood after exercise.
2	113.0	101.5	100.9	Old beef blood.
2	47.6	47.9	47.6	Lactic acid added to above blood.
	160.6	148.4	148.5	Blood and lactic acid by addition.
2	157.3	145.3	145.8	“ “ “ “ determined.

therefore, that the sulfuric acid reaction yields bisulfite-binding substances not coming from lactic acid. It might also be expected from the work of Schneyer (10) and Maver (6) on urine, when the method of Meissner (8) (collection and volumetric measurement of carbon monoxide liberated) is used, that blood, under such treatment, would yield carbon monoxide from other sources. However, the fact that the results check so closely with those by permanganate oxidation is good evidence that we are here determining end-products of the breakdown of the same substance, lactic acid. The chief interfering substances found by Maver in urine were hippuric, diacetic, and oxalic acids. Diacetic acid can be removed by boiling with a mineral acid. Our customary method with blood

filtrates has been aeration for 20 minutes in a boiling water bath after acidifying the solution, which removes traces of diacetic acid as well as acetone. Oxalic acid may be removed by treatment with calcium chloride and ammonia. It is also removed in the copper sulfate-calcium hydroxide precipitation as is our routine procedure.  $\beta$ -Hydroxybutyric acid and fatty acids yield only minute amounts of carbon monoxide by this method, but it is present in but small quantities in blood filtrates. It seems probable that the sulfuric acid method ordinarily yields results on tungstic acid blood filtrates about 10 mg. per cent too high. It is of interest to note that in blood of the same individual containing normal and high amounts of lactic acid (after exercise), the difference between results by the two methods is the same for both bloods. The amount with the sulfuric acid method is 10 mg. per cent too high with one subject and 13 mg. per cent with another. Two bloods with a high non-protein nitrogen gave the usual differences between results for lactic acid by the two methods.

*Retention of Lactic Acid by Protein Precipitation.*

In this series and in all following experiments, the permanganate oxidation method has been used. The blood proteins are precipitated in a 1:10 dilution. The sugar is removed by precipitation with copper sulfate and calcium hydroxide, making a total dilution of 1:15. An amount of filtrate is used sufficient to give aldehyde equivalent to from 0.2 to 0.3 mg. of lactic acid. This would be filtrate equivalent to 1 or 1.5 cc. of a normal blood. The following methods of precipitation have been used.

1. *Tungstic Acid*.—1 volume of blood, 8 volumes of 1/12 N  $\text{H}_2\text{SO}_4$ , 1 volume of 10 per cent sodium tungstate. Filtration.

2. *Mercuric Chloride*.—1 volume of blood, 7 volumes of 2 per cent  $\text{HCl}$ , 2 volumes of 5 per cent  $\text{HgCl}_2$ . Filtration.  $\text{Hg}$  is removed by  $\text{H}_2\text{S}$ ;  $\text{H}_2\text{S}$  is removed by aeration plus 1 drop of 20 per cent  $\text{CuSO}_4$  before final filtration.

3. *Mercuric Nitrate*.—1 volume of blood, 6 volumes of  $\text{H}_2\text{O}$ , 1 volume of  $\text{Hg}(\text{NO}_3)_2$  solution. (In 1 liter, 160 cc. of concentrated  $\text{HNO}_3$ , 220 gm. of  $\text{HgO}$ , 60 cc. of 5 per cent  $\text{NaOH}$ .)  $\text{Hg}$  is precipitated by  $\text{NaHCO}_3$ . Filtration.  $\text{Hg}$  is removed by  $\text{H}_2\text{S}$  and 1 drop of  $\text{CuSO}_4$  before final filtration.



4. *Metaphosphoric Acid*.—1 volume of blood, 8 volumes of  $H_2O$ , 1 volume of 10 per cent  $HPO_3$ . Filtration.

The data in Table IV show that within the limits of error inherent in the lactic acid method, the results with the filtrates from mercuric chloride and from metaphosphoric acid precipitations check with the tungstic acid filtrates. The results by the mercuric nitrate method, however, are variable and nearly always lower, 36 per cent in some cases.

To learn whether the apparent loss of lactic acid by mercuric nitrate-bicarbonate precipitation is actually lactic acid or some

TABLE IV.  
*Lactic Acid in Blood Filtrates.*

Results expressed as mg. per 100 cc. of blood.

Precipitation No.	Method of precipitation.				Blood.
	Tungstic acid.	$Hg(NO_3)_2$	$HgCl_2$	$H_2PO_4$	
1	286.9	190.6			Old beef.
2	71.7	52.2		71.0	Beef.
3	70.0	56.5		70.6	"
4	120.0	94.0	120.8		Human.
5	69.4	65.5			"
6	116.0	94.0			Beef.
7	117.6	87.8			"
8	30.2	19.4	31.2	30.9	Human.
9	30.1		31.8		"
10	35.9		34.6		"
11	92.8	90.0	93.0	92.0	Sheep.

other interfering substance, a series of determinations has been made on blood and on casein solutions to which known amounts of lactic acid are added before precipitation of the protein, on the supposition that the lactate ion of the zinc lactate is retained in the same proportion as the lactate ion normally present. The results (Table V) show a definite retention of the added lactate. The fact that mercuric nitrate-bicarbonate is the most efficient reagent for the removal of protein products of hydrolysis preliminary to total carbohydrate determinations prompted further investigation of its usefulness as a precipitant in lactic acid analyses. Since several cases where it has been used as a precipitant have given

theoretical yields of lactic acid, it seems possible that control of conditions under which it is used might lead to consistent results. It should be noted in this connection that the filtrate from tungstic acid precipitation is slightly acid (pH 4), the filtrates from the metaphosphoric acid and the mercuric chloride precipitations are strongly acid, in contrast with those from the mercuric nitrate-bicarbonate, which are alkaline. This suggests the possibility that our failure to recover lactic acid quantitatively from mercuric nitrate-bicarbonate precipitation is due to the alka-

TABLE V.

*Loss of Lactic Acid by Precipitation of Blood by Mercuric Nitrate-Bicarbonate Method.*

Results expressed as mg. of lactic acid per 100 cc. of blood.

No addition.	With added lactic acid.	Lactic acid recovered.	Lactic acid added.	Per cent lost.
35.9*	93.0	54.0	97.3	44.5
35.9	104.0	104.0	196.0	47.0
35.9	190.1	190.0	290.0	34.5
90.0	118.8	28.8	50.1	42.5
90.0	162.0	72.0	101.5	29.1

*5 Per Cent Casein with Added Lactic Acid.*

Results expressed as mg. of lactic acid per 100 cc. of solution.

Lactic acid added.	Lactic acid recovered.	Per cent lost.
20	15.6	22.0
40	33.2	17.0
80	65.4	18.2
100	84.4	15.6
200	158.0	21.0

\* Tungstic acid filtrate contained 39.5 mg. per 100 cc. of blood.

linity of the solution. This may be due either to the possible oxidation by the mercuric ion of some of the lactic acid, or by adsorption. If the loss is due to oxidation, the variations in percentage might be explained by variations in time and in alkalinity of the solutions. If, on the other hand, it is an adsorption phenomenon, we would expect variations in the amounts retained. The possibility of the protein carrying some of the lactate ion down as it is precipitated is untenable, since in alkaline solution the protein

acts as an acid and is in the form of sodium proteinate. On this basis a retention of lactic acid would be much more apt to occur when acid precipitants are used, especially precipitants of only moderate acidity as tungstic acid. This, however, is not the case. The stronger acid is efficient in replacing the weak acid.

The possibility of destruction of the lactate ion in alkaline solutions of mercuric nitrate has been investigated. To solutions of zinc lactate, mercuric nitrate solution is added and precipitated by the addition of sodium bicarbonate, until just alkaline to litmus. If the precipitation is carried on rapidly as for blood, quantitative recovery is accomplished. If, however, it is allowed to stand for a period of 3 hours in alkaline solution, as much as 6 per cent of the lactic acid disappears. Since the lactic acid cannot be quantitatively recovered by making the solution acid before removing the mercuric ion, it is presumably destroyed. The other reagents were tried separately. Mercuric nitrate has no effect in reducing the yield of lactic acid if not made alkaline, nor has nitric acid. Aeration with hydrogen sulfide also has no effect.

Since 6 per cent of the lactic acid is lost from pure zinc lactate solutions, as compared with 36 per cent from blood filtrates under similar conditions, it seemed to us that the lactate ion was in some way adsorbed by the alkaline protein mercuric oxide combination. If alkalinity could be avoided, we might avoid loss of lactic acid. The greater efficiency of mercuric nitrate-bicarbonate as compared with mercuric chloride as precipitants of protein we ascribed to two factors: first, the difference in pH under which precipitation is carried on, alkalinity favoring complete precipitation with the positive mercuric ion; second, mercuric nitrate is a highly ionized salt, while mercuric chloride is one of the exceptional salts which is but little ionized. This difference in concentrations of the precipitating mercuric ion, independent of changes in acidity, should be a factor in altering efficiency. These assumptions have been substantiated by experiments. When we use mercuric nitrate without bicarbonate neutralization, but remove excess mercuric ion directly with hydrogen sulfide precipitation, we obtain, when using unhydrolyzed blood, a yield of lactic acid only 2 per cent lower than on tungstate filtrates of the same blood, as compared with 36 per cent lower in some cases when alkali was used. On applying this procedure to blood that has been hydrolyzed for 3

hours with 0.5 N hydrochloric acid, we obtain clear filtrates, but they give a strong biuret reaction and the aldehyde yields are variable and in no way check with the lactic acid known to be present.

More careful adjusting of the alkalinity has been tried. If carried out with sodium bicarbonate as has been done up to this time, the constant evolution of carbon dioxide makes it impossible

TABLE VI.

*Effect of Varying pH at Which Mercuric Oxide Is Precipitated on Yield of Lactic Acid from Blood and Blood with Known Amount of Added Lactic Acid.*

Lactic acid added, 77.6 mg. per 100 cc. Results expressed as mg. of lactic acid per 100 cc. of blood.

Precipitation method.	pH	Lactic acid found.		Added lactic recovered.	Per cent recovered.
		Blood.	Blood plus added lactic acid.		
Tungstic acid filtrate.		37.8	111.7	73.9	95.2
Hg(NO <sub>3</sub> ) <sub>2</sub> , H <sub>2</sub> S.	Acid.	37.6	111.7	74.1	95.4
Hg(NO <sub>3</sub> ) <sub>2</sub> , HgO precipitated by NaOH, remaining Hg removed by H <sub>2</sub> S.	5.0	37.8	111.5	73.7	95.0
	5.6	37.7	111.6	73.9	95.2
	6.0	37.6	111.8	74.2	95.6
	6.5	37.9	111.7	73.8	95.1
	7.0	37.7	111.6	73.9	95.2
	7.5	33.8	90.3	59.5	75.8
Hydrolyzed blood precipitated as above.	7.0	36.8	111.8		
	7.0	37.4	110.9		
	7.0	37.6	111.7		
	7.0	37.7	111.6		
Average.....		37.4	111.5	74.1	95.0

to adjust the pH with any degree of accuracy. Acid or basic phosphate removes the mercury very efficiently as insoluble mercuric phosphate, but the results are not satisfactory, as the protein is not completely removed. The relative insolubility of borax makes it impossible to adjust rapidly the end-point. When sodium hydroxide is used and the solution is made up to volume after neutralization, a point can be reached when all proteins or their split-products which would yield a biuret reaction, are re-

moved, the lactic acid remaining in solution. This point is reached at a pH 7 when the solution is neutral to litmus. If blue litmus paper is used as an indicator, when it is no longer turned red the proper pH has been reached (Table VI).

Our observations with respect to retention of lactic acid are opposed to those of Mondschein (9) who found slightly more retention when he carried out the precipitation of egg albumen to which lactic acid had been added in acid solution than when he precipitated the mercuric ion in alkaline solution, though he reports retention in both cases.

The precipitation of the mercuric ion as oxide at a definite pH is necessary to remove completely protein fragments. When the mercuric ion is precipitated by phosphate at a similar pH, it is removed before sufficient alkalinity has been reached to carry the protein with it. It is of interest to note that on either side of a pH 7 some of the protein goes back into solution.

The best procedure for hydrolyzed blood is to add 4 volumes of water, allow time for laking, then slowly add 4 volumes of mercuric nitrate solution, care being taken to keep the precipitate finely divided by shaking or stirring. Titrate with sodium hydroxide of such strength that less than 2 volumes will be required to neutralize the acid present. Since the yellow precipitate of mercuric oxide makes the use of an added indicator impossible, spotting on blue litmus paper is the easiest way to determine the desired alkalinity. The volume is made up to a 1:10 dilution, well mixed, and filtered. If the correct pH has been reached the precipitation will be granular and filtration will proceed rapidly and the filtrate will be clear and colorless. If too alkaline, filtration is slow, the mercuric oxide precipitate is finely divided, and may go through ordinary filter paper, making the filtrate cloudy. If too acid, a drop of sodium hydroxide may be added and the solution refiltered. 1 drop of concentrated sulfuric acid should make a volume of 50 cc. of filtrate, acid to litmus preparatory to removal of the remaining mercuric ion by hydrogen sulfide. The hydrogen sulfide, in turn, is removed by aeration with air previously saturated with water, which process makes no measurable change in volume. Finally 1 drop of copper sulfate is added to remove any remaining traces of hydrogen sulfide, and the solution is filtered. A water-clear filtrate is obtained which gives no biuret reaction and con-

tains added sugar quantitatively and from 92 to 94 per cent of added lactic acid. This filtrate, after removal of sugar, may be used for the determination of lactic acid by the permanganate method, but not by the sulfuric acid method. Table VII shows a series of eight completely separate determinations on the same blood, with and without added lactic acid, precipitated by the tungstic acid and the mercuric nitrate methods.

Sugar determinations have also been included. These have been made by the Shaffer-Hartmann method, Somogyi's (11)

TABLE VII.

*Comparison of Lactic Acid and Sugar Yield as Determined in Tungstic Acid and Mercuric Nitrate Filtrates.*

Results expressed as mg. per 100 cc. of blood.

Tungstic acid nitrate.				Mercuric nitrate nitrate.			
Lactic acid.		Sugar.		Lactic acid.		Sugar.	
Blood.	Blood and added lactic acid.	Blood.	Blood and added sugar.	Blood.	Blood and added lactic acid.	Blood.	Blood and added sugar.
54.2	96.3	82.3	132.3	52.3	93.8	70.3	119.9
54.7	97.0	82.6	131.9	52.6	94.1	68.8	118.4
54.1	96.8	81.6	130.6	53.1	91.6	69.4	117.9
54.2	96.1	82.9	133.0	52.2	94.5	69.6	118.6
53.9	96.3	82.6	132.4	52.8	94.2	68.2	118.6
53.8	96.5	81.8	132.3	52.1	93.9	69.3	119.7
53.9	96.4	82.0	131.1	52.2	96.0	70.0	119.0
52.9	95.9	82.2	131.3	52.5	92.5	69.2	119.5
Average.....	96.4	82.3	131.8	52.4	93.8	69.3	118.9
Per cent recovered.....	94.5±3		99 ±2		93.2±3		99.1±2

modified solution being used, after adjusting to a pH of 6.8, just acid to phenol red. Although the recovery of added sugar is quantitative with both methods, the reducing substances in the filtrate of the blood precipitated by the tungstic acid method or mercuric chloride are 13 mg. per cent higher than by the mercuric nitrate method. These results are similar to those reported by Harned (4) using the Folin-Wu method for determining sugar. Since added sugar is not retained by mercuric nitrate precipitates,

it is evident that some other reducing substance is removed by mercuric nitrate but not by tungstic acid. It would follow from this that the alkaline mercuric nitrate precipitation is active in removing these substances. The fact that the nitrogen content of mercuric nitrate filtrates is extremely low as compared with the nitrogen content of tungstic acid and mercuric chloride filtrates, is evidence tending to show that the reducing non-sugar is a nitrogenous substance. No attempt has been made to show that all the reducing non-sugar has been removed.

*Effect of Change in Dilution While Precipitating the Protein.*

Blood precipitated in 1:5 and 1:10 and 1:20 dilutions, when tungstic acid, mercuric chloride, and mercuric nitrate (Table VIII)

TABLE VIII.

*Effect of Precipitation of Proteins in a More Concentrated Solution on Recovery of Lactic Acid.*

Method of precipitation.	Dilution during precipitation.			
	1:20	1:10	1:5	1:3
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Folin-Wu.....		286.9	227.9	
“ .....	55.9	54.8	48.2	
“ .....	56.8	56.0		
Mercuric nitrate.....		193.6	193.1	80.8
“ “ .....	54.2	54.9	53.8	45.3
“ chloride.....	56.8	55.9	54.3	

are employed, shows that lactic acid is retained to a greater extent when the protein is in high concentration, irrespective of the precipitant used. In the case of tungstic acid in a 1:5 dilution, the precipitate retains as much as 8 per cent of added lactic acid. A 1:10 dilution retains from 3 to 4 per cent, while a 1:20 dilution increases the yield but slightly, if at all. The other methods show less retention on concentration, but it is evident that less than 1:10 dilution is undesirable.

The further dilution of the protein-free filtrate necessary for the removal of sugar may be reduced by adding  $\frac{1}{10}$  volume of 25 per cent copper sulfate and enough dry calcium hydroxide to make the solution alkaline. The usual procedure requires  $\frac{1}{4}$  volume of

copper sulfate and  $\frac{1}{2}$  volume of a suspension of calcium hydroxide. This change gives a dilution of 1:11 instead of 1:15.

From the foregoing, it is clear that lactic acid may be determined in blood filtrates by the usual methods of precipitation. The determined lactic acid is between 93 and 94 per cent of that known to be present when a known amount has been added to blood. This includes the 2 to 3 per cent error inherent in the determination of the lactic acid when in pure solution. The recovery from purified casein solutions is of the same order of accuracy. The appreciable loss of lactic acid when separated from proteins by

TABLE IX.

*Comparison of Lactic Acid Recovery by Precipitation and Extraction Methods.*

Results expressed as mg. per 100 cc.

Method.	Tungstic acid.	Mercuric chloride.	Mercuric nitrate.	Meyerhof extraction.	Clausen extraction.
Blood.	85.6	85.3	84.3	84.3	83.6
	86.1	85.6	83.2	82.9	80.4
	84.9	84.2	82.1	85.1	85.1
	85.3	84.9	83.6	83.2	84.2
Average.....	85.5	85.0	83.3	83.9	83.3
Blood with 52 mg. lactic acid added per 100 cc.	135.4	134.2	132.1	133.4	132.2
	134.9	132.8	131.2	131.6	131.6
	135.3	133.1	132.0	132.3	131.8
	135.0	135.3	130.5	133.1	133.2
Average.....	135.2	133.9	131.5	132.6	132.2
Added lactic recovered...	49.7	48.9	48.2	48.7	48.9
Per cent recovered.....	95.5	94.0	92.7	93.7	94.0

precipitation methods makes it of interest to see whether a better recovery can be accomplished by extraction methods. Those suggested by Meyerhof (7) and by Clausen (1) have been tried. The continuous ether extraction of the tungstic acid filtrates suggested by Clausen removes quantitatively lactic acid. Meyerhof's (7) technique consists of precipitation of the proteins with alcohol and repeated washing of the protein coagulum and final evaporation of the combined washings and extraction of the water solution of this residue with amyl alcohol. When this technique



is employed, the lactic acid recovery is no greater than that obtained by the methods of protein precipitation already discussed. Table IX gives a summary of the results by the three precipitation methods and the extraction methods applied to the same blood with and without added lactic acid.

*Effect of Standing on Lactic Acid in Blood Filtrates.*

Standing for a period of 72 hours shows no effect on the lactic acid as determined by the permanganate method, either before or after removing the sugar or precipitated proteins. It should be noted, however, that we have occasionally observed a loss in lactic acid in the tungstic acid and metaphosphoric filtrates on standing. This has never occurred in mercury filtrates. Dr. Somogyi<sup>1</sup> finds that yeast precipitated with tungstic acid is still active, so presumably the loss in lactic acid which we have observed might be accounted for by some bacterial activity inhibited by mercuric salts but not by tungstate or metaphosphoric acid.

Standing in alkaline copper solutions has no effect at refrigerator temperature for a period of at least 72 hours, or at room temperature overnight.

SUMMARY.

Lactic acid may be determined in pure solution in quantities of the order of 0.2 mg. by the permanganate or sulfuric acid methods, if the aldehyde titration is used with an accuracy of  $98 \pm 3$  per cent.

The lactic acid of blood determined on tungstic acid filtrates is about 10 mg. per cent higher by the sulfuric acid method than by the permanganate method. The latter probably more nearly represents the true lactic acid content of the blood as the yield of carbon monoxide by the sulfuric acid method, assuming that its source is lactic acid, checks with the aldehyde by the permanganate method.

Considerable retention of lactic acid may occur with precipitation of the proteins. The more concentrated the protein solution, the greater the retention.

When mercuric nitrate precipitation is used, the pH at which

<sup>1</sup> Personal communication.

the mercuric oxide is precipitated has a marked effect not only on the completeness of protein precipitation, but also on the retention of lactic acid. A pH 7.0 represents the optimum point between failure of precipitation of protein on the acid side and greater loss of lactic on the alkaline side. From this filtrate 94 per cent of the lactic acid known to be present can be recovered as aldehyde.

The lactic acid remains constant in filtrates for as long as 72 hours at refrigerator temperature. Occasional changes have been noted in tungstic acid and metaphosphoric acid filtrates left at room temperature.

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# THE VOLUMETRIC ESTIMATION OF HYDROXYL GROUPS IN SUGARS AND OTHER ORGANIC COMPOUNDS.

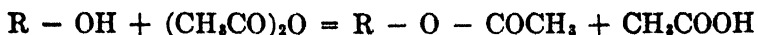
BY VERNON L. PETERSON AND EDWARD S. WEST.

(From the Department of Biological Chemistry, Washington University School  
of Medicine, St. Louis.)

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Many methods for the quantitative determination of hydroxyl groups in organic compounds have been described (1). The procedure most commonly used consists in the preparation of an acyl derivative (generally acetyl) and the estimation of the acid formed upon hydrolysis of it. Such a procedure requires a quantity of the material and the preparation and isolation of the acyl derivative in pure condition which is often difficult and laborious. The method of Tschugaeff and Zerewitinoff (2) based upon the Grignard reaction is applicable to small quantities but involves special apparatus and materials as well as being time consuming and inconvenient to execute.

Acetylation of hydroxy organic compounds with an excess of acetic anhydride in pyridine generally proceeds smoothly and to completion:



The acetic acid formed in the reaction is removed by combination with pyridine.

When the reaction product is poured into water the quantity of acetic acid available for titration is equivalent to the acetic anhydride used less that required for the acetylation of the hydroxyl compound. The presence of pyridine does not interfere with the titration of acetic acid to phenolphthalein. Consequently if a compound be acetylated and treated as outlined above and a blank prepared and treated similarly, equal quantities of acetic anhydride being used in both cases, the titration of the blank minus

that of the acetylation mixture represents the acetyl bound by the hydroxyl groups of the compound. This procedure was used for the determination of hydroxyl groups by Verley and Bölsing (3) especially in ethereal oils. Apparently, however, the method has been used much less than its merit justifies and its applicability has been tested on only a few compounds.

TABLE I.

	Sample.	Pyridine + anhydride.	Temperature.	Time.	Acetyl found.	Acetyl calcu- lated.	Per cent varia- tion.
	gm.	cc.	°C.	hrs.	gm.	gm.	
Glucose.....	0.2614	4	60	30	0.3084	0.3122	-1.2
Sucrose.....	0.1619	5	90	18	0.1601	0.1628	-1.7
Mannitol.....	0.1840	5	37	120	0.2668	0.2608	+2.2
Arabinose.....	0.1793	5	37	120	0.2063	0.2056	+0.3
Mannose.....	0.1085	5	37	120	0.1267	0.1295	-2.2
Erythritol.....	0.1800	5	80	18	0.2455	0.2539	-3.3
Rhamnose monohydrate.....	0.1703	5	80	18	0.1568	0.1606	-2.4
Xylose.....	0.1640	5	37	120	0.1854	0.1980	-1.4
Glucose cycloacetoacetic ester...	0.3393	7	45	70	0.2105	0.2127	-1.0
“ “ acid....	0.3513	12*	45	48	0.2405	0.2460	-2.2
Anhydro glucose cycloacetoacetic ester.....	0.3619	12*	52	16	0.1199	0.1215	-1.3
Anhydro glucose cycloacetoacetic acid.....	0.3196	12*	52	16	0.1229	0.1205	+2.2
Hydroquinone.....	0.6600	5	75	20	0.5178	0.5160	+0.3
Benzoin.....	0.7250	5	75	20	0.1366	0.1423	-4.0
Resorcinol.....	0.8270	5	75	20	0.6334	0.6465	-2.0
$\beta$ -Naphthol.....	0.2635	5	37	120	0.0798	0.0785	+1.7

The values given are representative of two or more analyses in all but two or three instances.

If the molecular weight of a compound is known the number of hydroxyl groups present in it may be easily calculated from the weight of acetyl taken up by it.

\* 2 cc. of acetic anhydride and 10 cc. of pyridine.

After considerable experience the writers have found the procedure to be not only as simple as titration itself but also reasonably accurate when applied to quite small quantities of various compounds. The method has been tested especially on sugars

and sugar derivatives and also on a few other hydroxylated compounds. The results obtained are summarized in Table I.

#### EXPERIMENTAL.

##### *A. Materials.*

*Pyridine.*—A C.P. grade was dried over barium oxide and distilled. 5 cc. added to 25 cc. of 1 N acetic acid should not change the titration value of the latter to phenolphthalein.

*Acetic Anhydride.*—Merck's reagent quality.

*Hydroxyl Compounds.*—Chiefly the best grade products of the Special Chemicals Company, Eastman Kodak Company, and Kahlbaum. Sucrose from the Bureau of Standards.

##### *B. Procedure.*

0.1 to 0.8 gm. of substance, depending upon the number of hydroxyl groups present, is weighed out in a small glass tube and placed in a Pyrex test-tube 16×150 mm. to which are added 3 to 5. cc. of acetic anhydride pyridine mixture (1 volume of anhydride and 2 volumes of pyridine) accurately measured. A blank is prepared at the same time. The tubes are either fitted with good corks and permitted to stand at 37°C. in the warm room or else with Hopkins condensers and heated in an electric air oven. A satisfactory oven may be prepared by placing electric lamps in the bottom of a tin can provided with a wood cover perforated with holes large enough to carry the test-tubes. In this way the tubes and not the condensers are heated. The temperature may be controlled by varying the number and power of the lamps used. Insulation of the oven with asbestos is recommended. We have used an oven of nine tubes capacity. Of course any convenient method of heating may be used. After acetylation is complete (24 to 48 hours at 60–80°C.) the product is mixed with approximately 200 cc. of ice water and titrated to phenolphthalein with 0.5 N NaOH. Blank titration — sample titration = equivalent of acetyl bound by hydroxyl.

1 cc. 0.5 N NaOH = 0.0215 gm.  $\text{CH}_3\text{CO}$  and 0.5 mm hydroxyl.

When the hydroxylated compound is an acid the titration values must be corrected by the amount of alkali neutralized by the quantity of acid used.

The solution should be thoroughly agitated during titration to prevent local rise of pH and saponification of the ester. Compounds which resinify and decompose appreciably when treated as outlined above should be run at a lower temperature for a longer time and the acetic anhydride diluted with a greater quantity of pyridine than there suggested. Ordinarily the reaction products become somewhat brown but this is of no practical consequence. In the case of quite stable substances the time required for a determination may be shortened greatly by acetylation at higher temperatures under reflux as recommended by Verley and Bölsing.

Compounds which form highly unstable acetyl derivatives cannot be successfully examined by this procedure. The quantity of pyridine used may be varied over wide limits according to the solubility of the substance being analyzed.

The conditions of time, temperature, etc., used in the above determinations are not to be considered as well established optima for the various compounds. In many cases the experiments were set up and titrated at the convenience of the worker. It is probable that in most cases the temperature could be materially increased and the time correspondingly shortened without sacrificing accuracy. Greater accuracy can be attained by using somewhat larger samples and correspondingly more acetic anhydride and pyridine.

The writers wish to thank Professor A. I. Kendall and Dr. T. E. Friedemann for kindly supplying some of the compounds used in this investigation.

#### SUMMARY.

The number of hydroxyl groups in many organic compounds can be easily determined by treatment with acetic anhydride and pyridine followed by titration. The method apparently works well with a variety of sugars and sugar derivatives.

Results are given of determinations carried out on hydroxylated compounds.

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# THE QUANTITATIVE DETERMINATION OF IRON IN TISSUES.

By R. P. KENNEDY.

*(From the Department of Pathology, the University of Rochester School of Medicine and Dentistry, Rochester, New York.)*

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The small amount of iron that is contained in the animal organism precludes the use of the well known volumetric or gravimetric methods. The procedure which is here proposed constitutes a simplification of the colorimetric methods hitherto suggested, and attains an accuracy limited only by the optical measurement of color. In summary it involves digesting organic matter in a mixture of perchloric and sulfuric acids, and diluting to known volume. An aliquot portion is treated with a concentrated solution of sodium sulfocyanate and the resulting ferric salt is extracted with amyl alcohol. The alcoholic solution is compared in a colorimeter with a similar solution developed from a known amount of iron.

Since the color of ferric sulfocyanate is so intense, a colorimetric method seems to be the logical procedure as a quantitative measure of iron. An interesting comparison can be made in this connection on hemoglobin solutions. If it be assumed that the depth of color of a blood solution is an accurate measure of hemoglobin then greater accuracy could be secured by examining colorimetrically the iron in the blood converted to ferric sulfocyanate. This relationship is expressed quantitatively in Fig. 1. A spectrophotometric absorption curve is plotted for a blood solution which contains 1.89 mg. of hemoglobin per cc. Superimposed on this is drawn a similar curve of the equivalent iron (0.0063 mg. of Fe per cc.) as sulfocyanate. The area under each curve is an index of total color and it is found that iron combined as ferric sulfocyanate has more color by about 10 per cent than the same amount of iron combined as hemoglobin.

## EXPERIMENTAL.

*Standard Iron Solutions.*—0.5 gm. of “analytical” iron wire are weighed and dissolved in 10 per cent sulfuric acid. After complete solution, about 3 cc. of concentrated nitric acid are added to convert all the iron to the ferric condition. The solution is then made up to the required volume. It is convenient to have several standards corresponding to 0.1, 0.2, and 0.5 mg. of Fe per cc.

*Digestion.*—1 cc. of blood (or a weighed piece of tissue) is measured into a 100 cc. Kjeldahl flask. 5 cc. of concentrated sulfuric

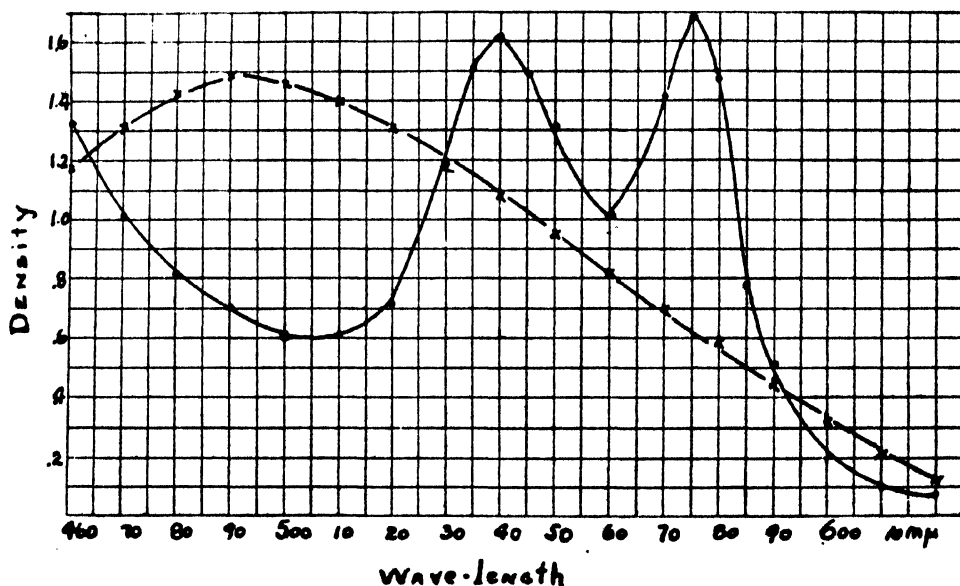


FIG. 1. Solid line represents the absorption curve of the hemoglobin solution. Broken line represents the equivalent iron as sulfocyanate in amyl alcohol.

and 2 cc. of 60 per cent perchloric acids are added and the material digested over a low flame. In about 10 minutes the digestion mixture is colorless and the completion is detected by a rapid ebullition and escape of oxides of nitrogen with subsequent slow boiling and the appearance of characteristic dense fumes of  $\text{SO}_3$ . After cooling, a drop of nitric acid is added and the mixture diluted to 100 cc. If the above procedure is carried out on a measured amount of the standard iron solutions, it will serve as a comparison standard in the colorimetric part. This compensates for the iron

in the reagents used. Also, which is equally important, the acidity of the standard and unknown solution is approximately the same.

*Colorimetry.*—10 cc. aliquot portions of the solutions (standard and unknown) are measured into 50 cc. glass-stoppered cylinders. 10 cc. of amyl alcohol and 5 cc. of a 20 per cent sodium sulfocyanate solution are added and the mixture is shaken at once. The alcohol layer separates sharply and almost immediately and contains quantitatively the colored ferric sulfocyanate. The colored layer is removed with a pipette and transferred to a colorimeter

TABLE I.

*Blood Analyses. Hemoglobin Calculated in Gm. per 100 Cc.*

Sample No.	Gas analysis. Volume per cent O <sub>2</sub> × 0.746.	Spectrophotometric analysis. Density × 11.65.*	Iron analysis. Gm. Fe + 0.00334.	Differences found in iron with O <sub>2</sub> .	Differences found in iron with color.
	gm.	gm.	gm.	per cent	per cent
1	20.40	20.50	20.10	−1.5	−2.0
2	19.60	19.90	19.70	−0.5	−1.0
3	14.52	14.65	14.70	+1.4	+0.3
4	12.45	12.85	12.55	+0.8	−2.4
5	17.90	18.05	17.96	0.0	−0.5
6	16.90	17.40	17.00	+0.6	−2.3
7	12.80	13.40	13.20	+3.0	−1.5
8	17.38	17.80	17.42	+0.3	−2.2

\* Kennedy, R. P., A spectrophotometric study of blood solutions, *Am. J. Physiol.*, 1927, lxxix, 346.

cup and compared with the standard in a Duboscq colorimeter. A green filter in the eyepiece secures greater accuracy in the color comparison.

This method has been applied to hemoglobin analysis of blood solutions and the results are included in Table I. For comparison analyses on the same samples of blood were made by the oxygen capacity and spectrophotometric methods. The accuracy of the iron method compares favorably with either of these. Included in Table II are some supplementary experiments showing the effect of phosphate, the addition of known amounts of iron, etc. The iron analysis of tissues is carried out in a manner similar to the

procedure outlined, except that where the total iron is small, the volume of solutions must be changed to effect a greater concentra-

TABLE II.  
*Triplicate Iron Analyses on Blood Samples.*

Sample No.	Fe per cc. blood found.	Hemoglobin per cc.	Maximum difference.
	<i>mg.</i>	<i>gm.</i>	<i>per cent</i>
6	0.564	0.1690	0.6
	0.568	0.170	
	0.568	0.170	
7	0.441	0.1320	1.8
	0.441	0.1320	
	0.449	0.1345	
8	0.577	0.1728	1.5
	0.586	0.1755	
	0.582	0.1743	

*Recovery of Added Iron and Effect of PO<sub>4</sub>.*

Solution.	Fe per 100 cc.	Additions per 100 cc.	Total iron in 100 cc.
	<i>mg.</i>		<i>mg.</i>
From liver.	0.191	0.10 mg. Fe.	0.293
" "	0.203	0.10 " "	0.304
" "	0.203	0.04 " "	0.240
" "	0.203	0.08 " "	0.284
" "	0.203	0.12 " "	0.330
" "	0.203	0.20 " "	0.404
" blood.	0.563	0.10 " "	0.660
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	0.50	K <sub>2</sub> HPO <sub>4</sub> ≈ 100 mg. P.	0.602
"	0.50	" ≈ 50 " "	0.546
"	0.50	" ≈ 10 " "	0.512
"	0.500	None.	0.504
"	0.250	"	0.252
"	0.500	1 cc. concentrated HNO <sub>3</sub> .	0.500
"	0.500	5 " " "	0.520

tion of iron. Forbes and Swift (1) recommend the use of iron-free instruments in obtaining tissue samples. In this work no material difference could be detected when bright instruments

were used or when the material was removed at autopsy by means of broken glass. Bits of tissue weighing up to 3 gm. can be digested in the sulfuric-perchloric mixture without previous mincing.

TABLE III.  
*Iron Content of Dog Tissues.*

Tissue.	Moist weight used.	Iron.
	gm.	per cent
Kidney, whole.....	1.216	0.0045
".....	0.766	0.0051
minced.....	4.455	0.0022
cortex. ....	1.460	0.0027
pyramid.....	1.654	0.0014
whole. ....	1.162	0.0168*
Spleen, whole.....	0.492	0.0826
".....	1.072	0.1020
".....	0.835	0.0250
".....	1.035	0.0215
".....	0.515	0.1620*
Liver, whole.. ....	1.239	0.0201
".....	1.156	0.0318
".....	1.160	0.0126
".....	2.291	0.0068
".....	0.920	0.0177*
Brain, whole.....	1.745	0.0025
".....	3.330	0.0016
Heart muscle.....	2.830	0.0042
Skeletal muscle.....	1.871	0.0059
"    ".....	2.700	0.0058
"    ".....	0.952	0.0051
"    ".....	1.440	0.0066*

\* Not perfused.

The data in Table III are derived from some preliminary experiments on perfused dogs (Whipple (2)) which serve to indicate the order of magnitude that can be expected regarding the iron content in blood-free tissue. The animals used were all normal adult dogs

in apparent good health. The animals were perfused by a method described elsewhere (2), since the effect of residual blood in the organs on the iron content is quite obvious. If organs be perfused after death there is considerable variation in the amount of edema that takes place and then the only practical way to express the iron is on the basis of percentage of fat-free dry weight (Magnus-Levy (3)). At first there occurred wide variations in the results obtained on duplicate samples of kidney which were occasioned by the method of sampling. It was found that the cortical portion contains almost twice the amount of iron as that in the pyramids. The variation in the iron of spleen and liver might be expected from the nature of the organ, although duplicate analyses varied not more than 4 per cent. In muscle, where the percentage is quite low, duplicate analyses varied occasionally as much as 10 per cent.

#### DISCUSSION.

The color of ferric sulfocyanate depends on the number of undissociated molecules, and a distinct advantage is to be gained by extraction with some organic solvent which removes the colored salt from the influences of acid, reducing salts, etc., which may be present. Natanson (4) suggested increasing the sensitivity of the sulfocyanate reaction for iron by extraction with ether. The underlying principle has been applied by Marriott and Wolf (5) who found that the addition of acetone improved the reaction. Lachs and Friedenthal (6) prefer the extraction with ether while several more recent writers, Berman (7), Fowweather (8), and Smirk (9), all use acetone and are mostly concerned with the problem of ashing. In any case, the data presented are none too convincing. Brown (10), Wong (11), and Elvehjem and Hart (12) overlook entirely the question of dissociation and continue to operate with aqueous solutions. The effect of light, temperature, acidity, and concentration on the reaction between ferric salts and sulfocyanates is admirably discussed by Philip and Bramley (13). The use of amyl alcohol in this work recommends itself (except for the odor) in that it removes quantitatively the ferric sulfocyanate from acid solutions, if the concentration of the iron salt is not too great. The alcohol is not very volatile and the volume after extraction is unchanged. The solutions are stable

towards light, but not heat, and the color obeys Beer's law. The effect of phosphoric acid in causing a fading of color has received much comment. From Table II it will be observed that a very large excess of phosphate increases the color to the extent of the iron impurity in the c.p. salt used. A trace of free nitric acid seems to be necessary in the reaction to offset the tendency of CNS ions to undergo oxidation which may cause a reduction in the iron.

The procedures hitherto suggested as a means of ashing probably constitute a matter of personal preference. The use of perchloric acid has undoubtedly been overlooked because of the impression which has been left by the explosive nature of the anhydride. In this work it has served as a most convenient and reliable reagent.

#### SUMMARY.

1. A method for the quantitative determination of iron in tissue is described.
2. Iron analyses on blood are correlated with oxygen capacity and colorimetric methods for hemoglobin.
3. Some data on iron content of tissue are given.

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# ON THE ACCURACY TO BE OBTAINED BY REPETITION OF SIMPLE MEASUREMENTS.

By AUGUST KROGH.

*(From the Laboratory of Zoophysiology, University of Copenhagen,  
Copenhagen, Denmark.)*

(Received for publication, January 5, 1927.)

The problem studied in the present paper is one which presents itself occasionally in biochemical work as in other sciences dealing with measurable quantities. Supposing the quantity to be measured to be near the minimum which can be read by the instruments available, is it possible materially to increase the accuracy by making a large number of repeated observations? In any well thought out measuring instrument of precision the minimum divisions correspond more or less to the limit set by the visual acuity (or power of discriminating shades of light or color) of a normal observer, and our problem can therefore be stated in the more general form: What can or cannot be gained by repetition of observations, which require the full utilization of the visual powers of the observer? The statement in this form shows that the problem is not simply statistical, but must be dealt with experimentally from a physiological point of view.

The writer was confronted with this problem in a very acute form in the results presented in a series of papers by Lundsgaard and Holbøll (1). These papers give the specific optical rotation of a substance called new-glucose, claimed to exist in blood and spinal fluid, to show a low specific rotation, but to have the property of mutarotation to  $\alpha$ ,  $\beta$ -glucose in just 48 hours at room temperature. The solutions on which the rotations were measured contained from 0.03 to 0.09 per cent glucose (in exceptional cases to 0.2).

The measurements were made by a technical saccharimeter read by means of a vernier scale to  $0.1^\circ$  Ventzke (corresponding for a tube of 200 mm. to 0.0328 per cent glucose). The initial readings (corresponding to specific rotations between  $16^\circ$  and  $42^\circ$ )

therefore often oscillated between  $0.0^{\circ}$  and  $0.1^{\circ}$ , and the final readings have very rarely exceeded  $0.3^{\circ}$ .

Nearly all the results have been obtained as averages of 40 readings, and it is claimed that such averages are reliable to at least the third decimal place. These claims are borne out by the results of determinations after 48 hours, when the specific rotation is on an average  $51.1^{\circ}$  with an average error of  $0.5^{\circ}$  (standard deviation =  $0.63^{\circ}$ ).

It is pointed out by the authors that the deviation of the readings from the theoretical value of  $52.5^{\circ}$  is due to the presence in the biological fluids of small and necessarily somewhat variable quantities of other substances having a low specific rotation. In several cases the mutarotation has been followed polarimetrically by a series of determinations. The curves obtained are so perfectly smooth that the single determination of specific rotation (40 readings) must be accurate to less than  $0.5^{\circ}$ , and in four cases the final rotation after 48, 52, 56 hours has been verified thrice, giving absolutely concordant results for the specific rotation; *viz.*, dialysate I,  $51.5^{\circ}$ ,  $51.5^{\circ}$ ,  $51.5^{\circ}$ ; dialysate II,  $51.7^{\circ}$ ,  $51.7^{\circ}$ ,  $51.7^{\circ}$ ; dialysate III,  $51.6^{\circ}$ ,  $51.6^{\circ}$ ,  $51.6^{\circ}$ ; spinal fluid  $50.2^{\circ}$ ,  $50.2^{\circ}$ ,  $50.2^{\circ}$ .

It appears therefore that the claim concerning a very high degree of precision to be obtained by simple repetition of relatively crude observations has been amply substantiated.

On the other hand this claim is certainly at variance with the practical experience of every trained observer, and it cannot but arouse some doubt that a number of obvious precautions have evidently not been taken by the authors according to their description of technique (2). The possibility of a correction for the zero point of the instrument used does not seem to have occurred to them. The readings are never carried beyond the first decimal place, though the vernier scale invites the use of the intermediate 0.05 and allows differences of 0.02 to 0.03 to be judged with considerable certainty. Of even greater importance than this technical point is the possible influence of self-suggestion on the part of the observer which is not mentioned at all in their description of the technique.

These considerations made it seem desirable to investigate more closely the claim for exceptional accuracy put forward, the more so as neither the statistical nor the more general problem presented

by such measurements seems to have been seriously considered before.

I shall deal first with the purely statistical aspect, on which I have had the benefit of consultation with several friends representing mathematical, physical, and biological sciences.

The reading of an ordinary saccharimeter is like other simple measurements composed of two procedures. The quartz wedge is adjusted until the fields appear perfectly equal, to the observer's eye, and the displacement from the zero position is read off. Supposing the adjustment to be absolutely correct while the reading is taken to the nearest line on the vernier scale, it is obvious that in practically all cases only one reading will be possible and there will be a maximum error on the result of  $\pm 0.05^\circ$  which will be

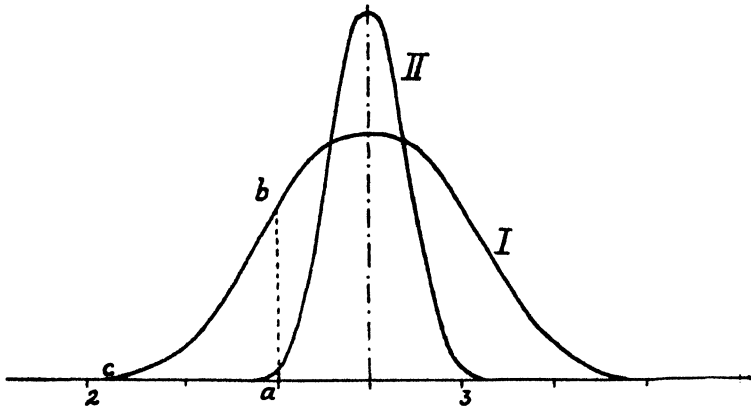


FIG. 1.

realized when the true value is just in the center between two possible readings. No amount of repetition will have the slightest influence. If the adjustment is liable to error, and supposing the errors to be distributed according to the exponential law, the result can be improved by repetition with the interesting consequence that the uncertainty of the final result can be definitely diminished by errors introduced into the adjustment. Supposing for instance in a case where the true value is 2.75 that the errors of adjustment are distributed in accordance with Curve I in Fig. 1, it is obvious that a certain proportion of the readings, corresponding to the area (*abc*), will give the value 2 while the rest will give 3. When a sufficient number of readings has been taken there will be

approximately 1 reading of 2 for every 7 of 3 and the result will be 2.88 instead of 2.75. If the adjustment is more precise corresponding for instance to Curve II, all the readings will be 3, but even in this case the uncertainty will be definitely reduced, since all true values below 2.75 and above 1.25 will cause distribution of the readings between 2 and 3, so that the maximum error will be nearly 0.25. Increased errors of adjustment will tend within certain limits to reduce the error of the average result. Supposing the number of readings to be constant ( $n = 40$ ) the errors of adjustment will cause an uncertainty (mean error) of the final result

TABLE I.

Thickness of Needle 18.

<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
0.7	0.7	0.8	0.7
0.7	0.7	0.8	0.7
0.8	0.8	0.7	0.7
0.7	0.8	0.8	0.7
0.7	0.7	0.7	0.8
0.7	0.7	0.8	0.7
0.7	0.7	0.7	0.8
0.8	0.8	0.7	0.7
0.7	0.7	0.8	0.8
0.8	0.7	0.7	0.7
Average.....0.73	0.73	0.75	0.73
General average.....	0.735		
Micrometer measurement.	0.725		

which is the standard deviation of each adjustment divided by  $\sqrt{n}$ . With a standard deviation of 0.5 the mean error of the final adjustment will be 0.08 and the errors due to the fact that the readings are distributed only between 2 or (at most) 3 whole numbers will only increase the uncertainty to about 0.1, and more generally it is found that standard deviations between 0.3 and 0.6 will result in the same final uncertainty of about 0.1 in a series of 40 determinations (corresponding in the actual determinations to  $0.01^\circ$ ).

It should be remembered, however, that this discussion is purely formal. It makes the arbitrary assumption that the errors of ad-

justment are *accidental* in the statistical sense, an assumption which the readings are too crude to prove or disprove.

In order to study the problem experimentally I have made a few series of measurements of well defined objects on which the actual deviation of the average result from the true value could be ascertained.

In the first experiments the thickness of cover-slips, microscope slides, and needles were measured by means of a caliper square divided in mm. and provided with a vernier which could easily be read to 0.1 mm. The measurements which were repeated

TABLE II.

Object.	No. of readings.	Average.	Micrometer reading.	Differences.
		<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
Cover-slip 1	10	0.22	0.203	-0.017
“ 2.	10	0.25	0.195	-0.055
“ 3.	10	0.20	0.173	-0.027
Slide 1.	10	1.00	0.956	-0.044
“ 2.	10	1.02	1.010	-0.010
Needle 0.	10	0.30	0.293	-0.007
“ 1.	10	0.36	0.320	-0.040
“ 5.	10	0.50	0.457	-0.043
“ 9.	20	0.695	0.642	-0.053
“ 19.	40	0.735	0.725	-0.010

Highest difference, -0.055. Lowest difference, -0.007.

Average difference calculated for 10 readings, -0.028.

10 to 40 times were checked afterwards by a screw micrometer. I have given one series of measurements in full (Table I).

The results of all the measurements are given in Table II, showing that the caliper square will always give too high results (as was to be expected) and on an average 0.03 mm. too high. It follows that in using the instrument every precaution should be taken to make certain that the arms are in good contact with the object, while it is useless to repeat the measurements more than a few times.

In the next series appropriate markings were made on a slide placed under the microscope and measured by means of a finely pointed needle fixed to the transverse limb of the mechanical stage.

The needle point was placed in contact alternately with the two ends of the object, and the scale and vernier read at each contact. Afterwards the length of the object was measured by a screw micrometer eyepiece, the measuring thread of which was arranged so as to be parallel with the corresponding limb of the mechanical stage.

As objects I have used short lines cut with a diamond (care being taken to select only lines showing sharply defined ends), drops of sealing wax melted onto the glass and showing a sharply defined elliptical outline, and drops of mercury. The micrometer was

TABLE III.

Length of line on glass. Naked eye adjustment, vernier readings.						Lens adjustment.			
Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.
25.8	26.4	25.8	26.2	25.8	26.3	25.8	26.3	25.775	26.25
25.7	26.3	25.7	26.2	25.8	26.3	25.7	26.3	25.70	26.25
25.8	26.3	25.8	26.3	25.8	26.3	25.7	26.3	25.725	26.30
25.8	26.4	25.8	26.3	25.8	26.4	25.8	26.3	25.75	26.30
25.8	26.3	25.8	26.3	25.8	26.3	25.8	26.3	25.80	26.30
25.8	26.4	25.8	26.3	25.8	26.3	25.8	26.3	25.775	26.30
25.8	26.3	25.7	26.3	25.9	26.3	25.8	26.3	25.775	26.275
25.7	26.4	25.8	26.3	25.8	26.2	25.8	26.3	25.75	26.275
25.8	26.4	25.8	26.3	25.8	26.3	25.8	26.3	25.75	26.275
25.8	26.3	25.7	26.3	25.8	26.3	25.8	26.3	25.775	26.30
25.78	26.35	25.77	26.28	25.81	26.30	25.78	26.30	25.756	26.283
Length... 0.57		0.51		0.51		0.52		0.527 mm.	

Micrometer reading, 793 = 0.534 mm.

tested by measuring on the Zeiss 1 mm. scale. 0.1 mm. was found to correspond at the magnification employed to  $148.5 \pm 0.5$  scale divisions on the drum of the micrometer or 100 scale divisions =  $0.0674 \pm 0.0003$  mm. The possible errors on the mm. scale are too small to influence the results in any significant way.

As an example of the results I give the readings on Line 3 where the contact with the needle tip was first observed with the naked eye (30 observations) and then with a lens magnifying 6 diameters (20 observations). In the last 10 observations the vernier was read very carefully judging to  $\frac{1}{4}$  or 0.025 mm. (See Table III.)

TABLE IV.

Object measured.	No. of measure- ments.	Average length. (A)	Mean of groups.	Deviation from mean.	Micrometer reading. (M)	Difference (M - A).
		mm.	mm.	mm.	mm.	mm.
Line 1.	10	0.70			0.597	-0.103
Line 2.	10	0.44		-0.017		-0.004
	10	0.47	0.457	+0.013	0.436	-0.034
	10	0.46		+0.003		-0.024
Line 3.	10	0.57		+0.047		-0.036
	10	0.51	0.523	-0.013	0.534	+0.024
	10	0.49		-0.033		+0.044
Line 4.	10	0.64		+0.005		-0.100
	10	0.63	0.635	-0.005	0.540	-0.090
Line 5.	10	0.41		+0.005		-0.070
	10	0.40	0.405	-0.005	0.340	-0.060
Sealing wax, Drop I.						
Observer R.	10	0.80		+0.040		-0.020
	10	0.88	0.840	-0.040	0.780	-0.100
Observer K.	10	0.67		+0.010		+0.110
	10	0.65	0.660	-0.010	0.780	+0.130
	10	0.66		0		+0.120
	10	0.75			0.780	+0.030
Sealing wax, Drop II.	10	0.34		-0.010		+0.067
	10	0.36	0.350	+0.010	0.407	+0.047
Mercury drop.	10	0.39		-0.003		+0.044
	10	0.41	0.393	+0.017	0.434	+0.024
	10	0.38		-0.013		+0.054
Numerical sum.....				0.299		1.335
Average deviation.....				0.015		0.061

All the measurements made by means of this technique have been tabulated in Table IV. This table shows generally a good agreement between successive series of 10 measurements of the same object. In spite of this agreement the average is often far



from correct. This shows that the adjustment of the needle to either end of the object can be duplicated with a fair degree of precision, but is at the same time influenced by a personal error of judgment which remains more or less constant during the series of measurements of the same object, but will show variations with different objects and undoubtedly also with the same object when conditions are changed. It is worthy of note that the same object (sealing wax, Drop I) measured by two different observers under identical conditions gave very discordant results: observer R. 0.84 mm., observer K. 0.66 mm.; true value 0.78 mm.

When the measurements of each object are considered separately it is seen that repeated series of 10 readings differ on an average only 0.014 mm. from their respective means, while the average difference from the true values is 0.062 mm. It is therefore useless

TABLE V.

Error.	Groups of 10 readings. Number of groups, 22. Probable error, $\eta = 0.052$ mm.		Groups of 5 readings. Number of groups, 44. Probable error, $\eta = 0.052$ mm.		Theory demands.
	Number.	Per cent.	Number.	Per cent.	
$< \frac{1}{2} \eta$	6	27	9	20	<i>per cent</i> 26.4
$< 1 \text{ "}$	11	50	23	52	50.0
$< 2 \text{ "}$	19	87	39	89	82.3
$< 3 \text{ "}$	22	100	44	100	95.7
$< 4 \text{ "}$					99.3

to repeat such measurements more times than is necessary to obtain an average reliable within this figure. This conclusion is borne out if groups of 5 readings are compared. The average deviation from the true value is in this case substantially the same as for 10 readings. I have examined the distribution of the errors in this series of measurements to see whether it agrees with the exponential law. This law demands a very definite distribution which can be approximately expressed by the probable error  $\eta$ . The probable error is equal to 85.4 per cent of the average error and is probable in the sense that half the number of errors should be smaller than  $\eta$  and the other half larger. Table V shows the agreement between theory and observations which cannot of course be expected to be complete when the number of observations is small.

Finally I have made a number of tests on polariscopes of the model used by Lundsgaard and Holbøll; *viz.*, the Schmidt-Haensch technical saccharimeter adapted for tubes up to 220 mm. length lighted by gas (Auer burner) or electric light, and provided with a light filter with bichromate solution.

TABLE VI.  
*Adjustments of Zero Position.*

Apparatus I. Vernier read by K.						
Observer.....	K.	R.		D.		J.
	-0.03	-0.10		+0.05		-0.07
	-0.05	-0.07		-0.13		-0.05
	-0.10	-0.10		-0.05		0
	0	0		-0.10		+0.07
	-0.03	0		-0.07		+0.10
	-0.05	-0.05		+0.05		+0.07
	0	-0.07		-0.05		+0.10
	0	-0.05		+0.07		+0.05
	-0.05	-0.03		-0.20		+0.10
	-0.03	0		+0.10		-0.03
Average.....	-0.034	-0.047		-0.033		+0.034

Apparatus A II.				Apparatus B II.		
Observer.....	K.	R.	H.	K.	R.	G.
	+0.06	+0.22	+0.13	-0.02	-0.1	0
	+0.04	+0.04	+0.10	+0.08	0	+0.1
	+0.06	+0.12	+0.10	+0.02	0	0
	+0.06	+0.10	+0.12	+0.10	0	0
	0	+0.12	+0.10	+0.16	+0.2	0
	+0.02	+0.10		+0.12	-0.1	-0.1
	+0.06	+0.20		+0.12	+0.1	0
	+0.04	+0.04		0	+0.15	0
	+0.04	+0.08		+0.08	-0.1	0
	+0.02	+0.10		0	-0.1	0
Average.....	+0.040	+0.112	+0.110	+0.066	+0.005	0.00

The adjustment of the instrument consists in turning a milled head moving the quartz wedge until the field of vision shows uniform intensity. In one of the instruments the field was divided in two by a vertical line (I). In two others there were two vertical

lines (II) and three fields, making the accuracy somewhat higher. Apparatus I and A II, for the loan of which I am indebted to Mr. Nyholm, chief of laboratory, Danish Sugar Refineries, had very clear and sharp fields, were easy to read, and caused us very little fatigue. Apparatus B II, which Professor Lundsgaard kindly allowed us to inspect, showed with the illumination employed a much darker and less well defined field, the adjustment of which we found rather difficult. This may be due however to our lack of familiarity with the somewhat unusual form and disposition of the illumination employed.

The position of the compensating wedge is read off on a scale and vernier viewed through a small microscope. The scale is divided in Ventzke degrees, and the vernier reads directly to  $0.1^\circ$ , but the lines are so sharp that subdivisions to one-fourth or one-fifth of this can be judged with considerable accuracy. In all instruments of this type and indeed in most instruments where the adjustment depends upon judging the equality in color or shade of parts of the field of vision, there is the serious drawback, strongly emphasized by J. S. Haldane in the case of colorimeters, that differences in the sensitivity of different parts of the retina will cause systematic variation in the results which it would be possible to avoid if the fields of the instrument could be reversed.

These differences come out very clearly in determinations of the zero point of the instrument which is not the same for different observers and shows irregular variations even for the same observer. This does not mean, of course, that the actual constants of the instrument undergo any change, but the variations are purely subjective and depend upon changes in sensitivity. Zero determinations for a number of different observers are given in Table VI, while examples of the variations encountered by the observer K. can be found in Tables VII and VIII.

The precision with which an adjustment of the field to uniformity can be reproduced has been studied on Apparatus I and A II by averaging groups of 5 readings which can be taken in such a short time (about 2 minutes) that retinal changes must at any rate be slight. On Apparatus I  $18 \times 5$  readings to  $0.025^\circ$  gave an average deviation of each adjustment from the mean of 5 of  $0.0443^\circ$ , corresponding to a standard deviation of  $0.056^\circ$ . The distribution of the errors follows very closely the exponential law. On Apparatus

A II  $28 \times 5$  readings to  $0.02^\circ$  gave an average deviation of  $0.0245^\circ$ , a standard deviation of  $0.0307^\circ$ , while the distribution was fairly good, the errors below  $\frac{1}{2}$  being 32 per cent instead of 26.4.<sup>1</sup> The superiority of the three-partitioned field is brought out clearly by the smaller deviations of the adjustment in Apparatus A II, though the difference is perhaps partly due to increased training on the part of the observer.

Solutions of pure cane sugar have been made up and introduced into polarization tubes of 200 or 220 mm. by Dr. Rehberg to whom I am also indebted for help on other points. Determinations of these unknown solutions were then made by the writer and the results compared with the figures calculated. One set of determinations was made according to the technique described by Lundsgaard and Holbøll (2), 40 readings to the nearest vernier division in the course of 15 to 20 minutes. Another set was made by taking alternately 5 readings on a tube filled with water (zero readings) and 5 readings of one of the solutions to be determined. This procedure was in each case repeated at least twice. Each determination consists in this case of 5 readings of the unknown corrected by  $5 + 5$  zero readings. All these were made as accurately as possible to  $\frac{1}{4}^\circ$  in the case of Apparatus I and to  $\frac{1}{8}^\circ$  in the case of Apparatus A II. On Apparatus B II 10 zero readings were taken, thereupon 10 readings of each solution, and then again 10 zero readings.

As examples of the readings obtained I give a series made with Apparatus A II in Table VII while the final results have been summarized in Table VIII.

These results show that without the application of a correction for the individual zero point the figures obtained by the Lundsgaard and Holbøll method of reading are not reliable in my case to more than about  $0.1^\circ$ . With a constant correction, different for each apparatus, but determined in each case on the same day or the day before, the results become much better, but even then there are discrepancies amounting on an average to  $0.019$ . 10 readings should be sufficient to obtain this degree of accuracy, and there is indeed some danger in making a larger number, because the retinal fatigue is likely to cause a shift of the zero.

<sup>1</sup> A good agreement is scarcely to be expected when the standard deviation comes close to the smallest interval read.

TABLE VII.  
*Readings on Apparatus A II.*

Solution I. 3.00-3.15					Solution II. 3.20-3.35					Solution III. 3.41-3.57				
0.1°	0.2°	0.2°	0.2°	0.2°	0.1°	0.2°	0.1°	0.2°	0.2°	0.3°	0.2°	0.2°	0.2°	0.2°
0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3
0.2	0.1	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.3	0.3	0.1	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3
0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.3	0.2
0.2	0.1	0.3	0.3	0.1	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2
0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2
0.16°	0.18°	0.23°	0.18°	0.18°	0.17°	0.19°	0.17°	0.18°	0.17°	0.21°	0.20°	0.20°	0.20°	0.23°
Average.....0.1875°					0.1775°					0.210°				
0	I	0	II	0	III	0	I	0	II	0	III	0	0	0
0.10°	0.16°	0.04°	0.12°	0.06°	0.16°	0.06°	0.20°	0.02°	0.20°	0.02°	0.18°	0.06°	0.06°	0.06°
0.12	0.18	0.04	0.20	0.02	0.20	0.04	0.18	0.06	0.18	0.10	0.22	0.04	0.04	0.04
0.10	0.24	0.04	0.12	0.06	0.20	0.06	0.10	0.04	0.20	0	0.18	0.06	0.06	0
0.02	0.22	0.08	0.24	0.04	0.20	0.06	0.12	0.04	0.14	0.08	0.18	0	0	0
0.04	0.18	0.10	0.18	0.06	0.22	0.00	0.16	0.02	0.20	0.04	0.18	0.06	0.06	0.06
0.076°	0.196°	0.060°	0.172°	0.040°	0.196°	0.044°	0.152°	0.036°	0.184°	0.048°	0.188°	0.044°	0.044°	0.044°
Rotation obtained.	0.128°		0.122°		0.154°		0.112°		0.142°		0.142°			

TABLE VIII.

Ventske degrees  $\times 1000$ .

Apparatus.	Rotation calculated.	Rotation observed, 40 readings to 0.1°.	Rotation corrected for zero error.	Difference (1) - (2).	Difference (1) - (3).	Rotation observed, 5 readings to $\frac{1}{10}$ or $\frac{1}{20}$ °.	Zero readings.	Rotation corrected for zero error.	Average rotation.	Difference (1) - (9).
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
I	114	33	135	+81	-21					
	277	210	312	+67	-35					
	380	267	369	+113	+11					
A II	235	348	253	-113	-18	300	58	242	236	-1
						328	98	230		
	225	330	235	-105	-10	280	50	230	223	+2
						304	88	216		
	121	188	92	-67	+29	204	70	134		
						220	90	130	126	-5
						196	68	128		
						152	40	112		
	112	178	83	-66	+29	212	68	144		
						208	70	138	136	-24
						172	50	122		
						184	42	142		
	121	210	115	-89	+6	228	76	152		
						212	62	150	150	-29
						196	42	154		
						188	46	142		
B II	(3900)					3970	66	3904	3900	
						3980	66	3894		
	390					432	66	366	360	+30
						420	66	354		
	39					108	66	42	30	+9
						84	66	18		

Still better results are obtained when the vernier is read as accurately as possible (to  $0.02^\circ$ ) while alternate determinations, each

of 5 readings, are taken of the unknown solution and the zero point. Even with these precautions the error may rise to  $0.03^\circ$  and is on an average  $0.012^\circ$  for the series given. Very little is gained by taking more than 5 readings for each solution. It would appear that some systematic error of unknown origin is present in the last determinations with Apparatus A II. Pressure on the cover-glasses of the polarization tubes might cause errors like these, but the cover-glasses were carefully adjusted so as to be under a minimum of pressure before each series.

In making very accurate determinations of dilute sugar solution the best plan would no doubt be to make alternate readings of the solution and a zero tube and this could be greatly facilitated if the instrument were so arranged that two tubes could be exchanged mechanically by moving a lever.

Returning briefly to the papers by Lundsgaard and Holbøll which have prompted the present study, it is necessary to state that while a purely theoretical reasoning puts the accuracy obtained in their polarimetric measurements at about  $0.01^\circ$  Ventzke, corresponding to  $1.7^\circ$  to  $3.5^\circ$  on the specific rotation of their dialysates from blood, the experimental investigation shows it to be even lower. When their determinations referred to above (p. 394) show an agreement which is several times as good ( $0.5^\circ$ ), it is hard to imagine how this result can have been obtained. I may add that experiments by Hagedorn (3) have failed absolutely to confirm the existence of a substance like the new-glucose postulated.

#### SUMMARY.

Some simple measurements have been studied to find out the degree of accuracy to be obtained by a large number of repetitions.

Measurements of lengths of about 1 mm. observed with the naked eye and read off by means of a vernier scale to 0.1 mm. cannot by repetition be made accurate beyond 0.05 mm. and a small number of repetitions is sufficient to insure this.

Weak sugar solutions having rotatory powers from  $1^\circ$  to  $0.1^\circ$  Ventzke measured polarimetrically on a vernier scale to  $0.1^\circ$  Ventzke cannot by repetition be determined beyond  $0.03^\circ$  Ventzke. When the vernier is read to fractions of  $0.1^\circ$  and alternate readings made of the zero point of the instrument, the accuracy can be pushed further and may possibly reach  $0.01^\circ$ .

The accuracy claimed in a series of papers by Lundsgaard and Holbøll for polarimetric readings repeated 40 times cannot be verified.

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# THE NATURE OF THE COMBINATION BETWEEN CERTAIN ACID DYES AND PROTEINS.\*

By ROSS AIKEN GORTNER.

(From the Division of Agricultural Biochemistry, University of Minnesota,  
St. Paul.)

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Chapman, Greenberg, and Schmidt<sup>1</sup> have recently published under the above title. Their paper contains data which they interpret as indicating that certain conclusions reached by Hoffman and Gortner<sup>2</sup> are invalid. However, their data appear to be capable of an exactly diametrically opposite interpretation and as such offer very striking substantiation of the conclusions reached by Hoffman and Gortner. It is the purpose of this communication to point out this interpretation and to leave the reader to draw his own conclusions as to which interpretation is correct.

Hoffman and Gortner, as the result of an extensive study of acid and alkali binding by proteins, drew the conclusion that the mechanism causing the binding of acid in the region lying between the isoelectric point of the protein and a hydrogen ion concentration equivalent to a pH of 2.5 was of a different nature than was the mechanism causing binding at hydrogen ion concentrations greater than pH = 2.5. Similarly, Hoffman and Gortner found that alkali binding between the isoelectric point of the protein and pH 10.5 differed from that at greater hydroxyl ion concentrations. The amount of acid bound at acidities lying between the isoelectric point of the protein and pH 2.5 was definitely correlated with the free amino nitrogen of the protein (one-half of the lysine

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<sup>1</sup> Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A., *J. Biol. Chem.*, 1927, lxxii, 707.

<sup>2</sup> Hoffman, W. F., and Gortner, R. A., Colloid symposium monograph, New York, 1925, ii, 209.

nitrogen) plus one-fourth of the arginine nitrogen. Accordingly in this region the acid binding was different for the various proteins and was dependent upon the chemical composition of the proteins. At greater acidities the further acid binding was independent of the chemical composition of the proteins and was related only to protein concentration. At acidities greater than pH 2.5 all proteins bound gm. for gm. the same amount of acid. Hoffman and Gortner studied systems containing at least 1.0 per cent of protein.

Chapman, Greenberg, and Schmidt have used much more dilute solutions, *e.g.* their gelatin solution was 0.172 per cent concentration, and find that: “. . . combination between the proteins and dyes used in these experiments takes place in stoichiometric proportions.” “The capacity of the proteins to combine with dyes can be correlated with the content of free basic groups of arginine, lysine, and histidine,” and “The data do not support the assumption that in acidities lying between pH 2.5 and 1.0 the combination between protein and dye is an adsorption phenomenon.”

Let us consider for a moment their experimental technique. In order to measure the dye-protein reaction they placed the protein “*in a solution of acid or alkali and the dye was then added.*”<sup>3</sup> If now we assume with Loeb that proteins react with hydrochloric acid to form a “protein chloride” which is highly ionized in solution, we would have, at least in certain of the acidities and certainly in the concentrations used by Chapman, Greenberg, and Schmidt, not protein plus dye but rather *positively charged protein micelles* (or protein cations) plus dye. However, Chapman, Greenberg, and Schmidt suggest that the dye is probably completely dissociated at pH 2.0. Thus we are no longer dealing with a dye molecule but rather with *negatively charged dye micelles* (anions) and  $H^+$ .

If now we have a positively charged ionic micelle (protein cation) and a colloidal (?) dye anion, it is obvious that mutual precipitation will occur, due to the neutralization of the electrokinetic potentials characteristic of surfaces. That such reactions should be stoichiometrical is not surprising, for *stoichiometrical reactions are a necessity if we consider that the  $\zeta$  potential on the surface must be reduced to zero before flocculation will occur and that when*

<sup>3</sup> The italics are ours.

*the  $\zeta$  potential is reduced to zero no further reaction between dye and protein can take place. Thus a constant amount of dye anion possessing a definite negative  $\zeta$  potential will flocculate a definite amount of positively charged protein possessing a constant positive  $\zeta$  potential<sup>4</sup> and the reaction will not necessarily be a reaction between primary valences but may be regarded as entirely an electrokinetic phenomenon.* These results, which we would expect from the colloid theory, are exactly the results observed by Chapman, Greenberg, and Schmidt.

Chapman, Greenberg, and Schmidt in their Figs. 4, 5, 6, and 7 show that a constant amount of dye is necessary to flocculate a given amount of protein. In Figs. 5 and 7 they include curves drawn from the data of Hoffman and Gortner in order to indicate how their curves differ from those of Hoffman and Gortner. It will be noted, however, that *the dye-protein flocculation curves are not a constant at hydrogen ion concentrations less than pH 2.5* but the equivalents of dye necessary to precipitate the protein (*i.e.* the equivalents necessary to reduce the  $\zeta$  potential to approximately zero) rapidly decrease toward the isoelectric point of the protein following, in so far as their few measurements show, very similar curves to the curves of Hoffman and Gortner. Their curve, Fig. 2, shows this exceptionally well, although here the curve is drawn only for the range of approximately pH 4.25 to 5.25.

Let us assume now that Hoffman and Gortner (p. 352) were

<sup>4</sup> It is thus only necessary to assume that the  $\zeta$  potential on the micelle increases up to a pH of 2.5 and thereafter remains constant. This would explain the rise in viscosity of protein sols which reaches a maximum at approximately pH 2.5 (*cf.* Gortner, R. A., and Sharp, P. F., *J. Physic. Chem.*, 1923, xxvii, 481, 567). The subsequent decrease in viscosity at greater acidities can be regarded as due to a dehydration of the ionic micelle without affecting the  $\zeta$  potential. We have already under way in this laboratory experiments which we hope will test this assumption.

It may be well to point out here that the  $\zeta$  potential is not the  $\epsilon$  or thermodynamic potential. The "membrane potentials" which Loeb measured were the  $\epsilon$  potentials and while these determine the thermodynamic equilibrium, they, aside from certain hydration effects, in no way affect the colloidal behavior of the system or colloidal stability. The  $\zeta$  potential, on the other hand, is concerned only with surface forces, and the magnitude and sign of the  $\zeta$  potential (together with hydration) determine the stability of colloid systems and colloidal behavior such as adsorption, flocculation, mutual precipitation, and all of the electrokinetic phenomena characteristic of colloids.

correct in stating that, "the chemical combination taking place between the isoelectric point of the protein (no acid or alkali bound) and pH 2.5 is due to the combination of acid with the free amino nitrogen of the native protein which is neutralized at about pH 2.8, and the sum of the free amino nitrogen and one-fourth of the arginine nitrogen which is neutralized at about pH 2.5," and that at greater acidities acid is no longer bound by definite chemical groups in the protein molecule but rather acid binding at acidities greater than pH 2.5 is solely determined by surface (colloid) forces.

This would mean that when a protein sol is acidified to a pH of 2.5 all of the protein is in the form of positively charged protein micelles (cations). It is obvious that if all of the protein is in the form of positively charged micelles at pH 2.5, no increase in acidity can produce a greater number of positively charged micelles; consequently any reaction between a charged protein micelle and a dye anion must reach a maximum value at pH 2.5 and be constant at greater acidities. This exactly accords with the data of Chapman, Greenberg, and Schmidt. The new colloidal micelle, produced by the interaction of protein and dye, is now neutral from the electrokinetic standpoint and should precipitate, which is according to the observations of Chapman, Greenberg, and Schmidt.

It is well known that dyes are *adsorbed* much more readily than are inorganic electrolytes. In this case, however, there is a tendency for the adsorption of hydrochloric acid at acidities greater than pH 2.5 (see data of Hoffman and Gortner) and it may well be that there is a slightly greater adsorption of the less mobile  $\text{Cl}^-$  than of the very mobile  $\text{H}^+$ , in which case the precipitated dye-protein floc will be slightly *negatively* charged in the higher acidities, and as such would not adsorb any additional dye anions, but would rather repel such an anion, causing the excess dye anions to remain in the mother liquor. This is exactly in accord with the data of Chapman, Greenberg, and Schmidt and in no way affects the conclusions of Hoffman and Gortner.

There is a large school of workers who insist that the reactions typical of colloid systems do not play a rôle in protein reactions; that protein reactions are stoichiometrical reactions and are governed only by the amphoteric nature of the protein which in turn

is dependent upon the basic and acidic groups of the protein molecule. These workers insist that adsorption is not a factor, but that the reactions are reactions of primary valence. Just why they should accept the view-point that aqueous systems of starches, celluloses, rubber, and other lyophilic or lyophobic systems may react as colloids but that proteins are characterized by an entirely different behavior, is incomprehensible. We know that the protein molecule is large enough to bring the size of particles well within the boundary of the colloid realm. We believe that, with the exception of the albumins, most proteins exist in "solution" as micelles. *If there are electrokinetic forces at interfaces between water and starch, cellulose, or glass, there must be electrokinetic forces at the interfaces of a protein-water system* and to ignore such forces is only to delay a full and complete understanding of protein behavior.

It is my belief that the data presented by Chapman, Greenberg, and Schmidt afford an unusually perfect substantiation of the conclusions drawn by Hoffman and Gortner.



## THE GLOBULINS OF RICE, *ORYZA SATIVA*.

By D. BREESE JONES AND CHARLES E. F. GERSDORFF.

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils,  
United States Department of Agriculture, Washington.)

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### *Globulins of Rice.*

Compared with the proteins of other cereals, those of rice have been but little studied, notwithstanding the fact that rice constitutes one of the most important sources of food for a large part of the world's population. One reason for this is doubtless due to the unusual distribution of the classes of protein found in this seed. Unlike the proteins of other cereals, nearly all of the proteins of rice endosperm are insoluble in water, salt solution, and alcohol, and belong to the rather ill defined class of proteins called glutelins. Consequently, most of the data recorded in the literature on rice proteins has been on a mixture of the total alkali-extractable proteins.

In 1908, Rosenheim and Kajiura (1), in a preliminary communication, announced that rice contained small amounts of an albumin (0.04 per cent) and of a globulin (0.14 per cent) which coagulated respectively at 85 and 70°C., and a relatively large amount of a protein having the properties of a glutelin to which they gave the name oryzenin.

Later (1909), Suzuki, Yoshimura, and Fuji (2) hydrolyzed the total alkali-soluble proteins of rice and also those of "rice bran," and determined the percentages of amino acids. The sum of the percentages of the amino acids found, however, amounted to only 44 per cent of the protein hydrolyzed in the case of the rice flour proteins, and 19 per cent in the case of the bran protein.

In 1915, Osborne, Van Slyke, Leavenworth, and Vinograd (3) extracted polished rice with 0.2 per cent sodium hydroxide solution, and precipitated the proteins with dilute acetic acid. The air-dried preparation obtained represented 4.17 per cent of the polished rice, and contained 16.68 per cent of nitrogen, calculated on an ash- and moisture-free basis. This protein was analyzed by the Van Slyke method. They also found a small amount of protein which was similar to gliadin with respect to its solubility in alcohol. On account of the small amount present, they were unable to determine whether it was an original constituent of the seed.

Later (1925), Hoffman (4) isolated from 7.5 kilos of polished rice 7.5 gm. of an alcohol-soluble protein which he analyzed by the Van Slyke method.



Tillmans and Alt (5) state that rice flour contains neither an alcohol-soluble, nor a water-soluble protein, and only a small quantity of a salt-soluble protein. They determined colorimetrically the tryptophane content of the globulin and also of the total mixed proteins, which they found to be 1.0 and 1.25 per cent respectively.

Recently, Kondo and Hayashi (6) in a series of articles reported a study of rice glutelin. Their glutelin contained 16.91 per cent of nitrogen. The distribution of nitrogen in the glutelin was determined and also the optimum conditions for flocculation of the protein.

The work outlined above represents, as far as we are aware, about all of the work that has been done on the nature and character of the different rice proteins. The results are not entirely in accord.

The work described in this article is concerned primarily, not with the glutelin, but with the other proteins of the rice kernel, which occur in small quantities, and concerning which we have but very little information. The rice used in our studies was from the 1924 crop of the Blue Rose variety grown in Louisiana.

By dialyzing saline extracts of white rice, we have been able to isolate a protein fraction consisting of two globulins, coagulating at 74 and 90°C. The concentrations of ammonium sulfate which precipitate these globulins are so close together that the globulins could not be separated by this method. They are both precipitated from a 5 per cent sodium chloride solution at about 0.3 of saturation with ammonium sulfate. The wide difference, however, between the temperatures at which they coagulate made it possible to separate them by fractional heat coagulation.

Careful examination of the dialysate from which the globulins had been removed failed to reveal the presence of any albumin. Tillmans and Alt (5) were likewise unable to detect the presence of albumin. The results reported by Rosenheim and Kajiura (1) wherein they claim to have isolated a globulin coagulating at 70° and an albumin coagulating at 85°, are very unusual and difficult to explain, as it is rarely if ever that an albumin has such a high coagulation temperature. These two coagulation temperatures agree so closely with those found for the globulins which we have isolated, as to indicate that they were dealing with two globulins rather than with a globulin and an albumin.

Aside from the difference in their coagulation temperatures, analyses of the globulins reveal marked differences between them in elementary composition and in the distribution of nitrogen.

The glutelin fraction of rice was prepared by extracting with alkali the white rice from which the globulin had been removed by means of sodium chloride solution, the method commonly used for preparation of glutelins. Inasmuch as the distribution of nitrogen and determination of most of the amino acids in rice glutelin prepared by this method has been already recorded by other investigators, only the results of colorimetric determinations of tyrosine, cystine, and tryptophane are here given. The analysis of rice glutelin prepared by a method recently devised in this laboratory (7), and by which it was shown that wheat contains two glutelins, forms the subject of the following article.

The isolation and study of the proteins of the branny seed coats of rice had been also contemplated, following the general methods which we have previously used for the study of the proteins of wheat bran. Inasmuch as we were unable to obtain rice bran free from the germ or embryo, a study was made of commercial rice bran which contains, besides the bran, practically all of the germ. A globulin fraction amounting to 2.88 per cent of the bran used, and a small albumin fraction were isolated. A fraction soluble in alcohol, having a rather indefinite composition, was also isolated. No protein of a glutelin type was found. The globulin fraction consisted of a mixture of apparently three globulins. On account of the indefinite character of the bran we were working with, no attempt was made to separate the individual proteins. As these proteins were derived almost entirely from the embryo and bran, the data presented are of value in throwing some light on the character of the proteins of those parts of the rice kernel.

### *White Rice.*<sup>1</sup>

For the extraction of the proteins of the rice endosperm, unpolished white rice was ground to a flour, the composition of which is given in Table I.

<sup>1</sup> The terms used to designate the rice products referred to in this article are explained as follows: white rice, the unpolished rice seed from which had been removed all of the seed coats and the embryo; brown rice, the seed containing the bran layers and embryo; rice bran, the milling by-product containing all of the bran, embryo, and some of the endosperm; prepared rice bran, the rice bran described above from which most of the endosperm had been removed.

Preliminary, half hour extractions at room temperature made on 5 gm. samples of the flour showed that 5 per cent sodium chloride solution was the most efficient concentration of this solvent. Boiling 70 per cent alcohol removed 0.028 (expressed in terms of the air-dried meal) per cent of nitrogen, equivalent to 0.17 per cent of crude protein.

In order to ascertain the total amount of nitrogen extractable from the flour by 5 per cent sodium chloride solution, 100 gm. of the flour were extracted by successive portions of salt solution until no more nitrogen was being removed. A total of 0.1654 gm. of nitrogen was extracted, equivalent to 17.50 per cent of the total

TABLE I.

*Nitrogen and Protein Content of White Rice, Brown Rice, Rice Bran, and Prepared Rice Bran.*

Material.	Moisture.	Ash.	Nitrogen.	Protein (N $\times$ 6.25).	Moisture- and ash-free.	
					Nitrogen.	Protein (N $\times$ 6.25).
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
White rice.....	11.61	0.37	0.93	5.81	1.06	6.63
			0.96	6.00	1.09	6.81
Brown " .....	11.32	1.12	1.21	7.56	1.40	8.75
			1.22	7.63	1.41	8.81
Rice bran.....	12.22	8.30	2.49	15.56	3.14	19.63
			2.45	15.32	3.08	19.25
Prepared rice bran...	11.96	12.74	2.49	15.56	3.31	20.69
			2.46	15.38	3.27	20.44

nitrogen of the flour. The joint saline extracts on heating to boiling gave 0.5025 gm. of a coagulum containing 0.1142 gm. of ash. Corrected for ash, this material represents 0.39 per cent of the flour equivalent to 5.75 per cent of the total calculated crude protein in the flour.

The total nitrogen extractable by alkali was determined by making eight successive, half hour extractions of 100 gm. of the flour with 200 cc. portions of 0.4 per cent aqueous sodium hydroxide until no more nitrogen was being removed. 68.46 per cent of the total nitrogen of the flour was thus extracted. Addition of tannic acid to the acidified extract precipitated 59.41 per cent of the total nitrogen of the flour.

An additional amount of nitrogen equivalent to 9.78 per cent was removed from the residue remaining after the exhaustive extraction with aqueous alkali, by further extracting the residue with boiling 60 per cent alcohol containing 0.4 per cent sodium hydroxide. Tannic acid precipitated from the resulting extract 1.45 per cent of the total nitrogen of the flour. The extractions with both aqueous and alcoholic sodium hydroxide removed accordingly 78.24 per cent of the total nitrogen of the flour. The nitrogen precipitable by tannic acid, amounted to 60.86 per cent of the total nitrogen of the flour.

### *Preparation of Globulins.*

Quantities of rice flour were extracted for 2 hour periods with 5 per cent sodium chloride solution, and the extracts filtered clear by suction through mats of filter paper pulp. Preliminary tests showed that when the extract was made 0.3 saturated with ammonium sulfate, practically all of the protein precipitable by that salt was separated. Further addition of ammonium sulfate, even to complete saturation, to the filtrate from the precipitate caused but a trace of material to separate.

Coagulation tests made on a solution of the protein precipitated by ammonium sulfate indicated the presence of two globulins, one coagulating at 74°C. and another at 90°C. Dialysis of either a solution of the protein precipitated by ammonium sulfate or of the sodium chloride extract of the flour, precipitated both globulins together; it left no coagulable material in the dialysate, showing the absence of any detectable amount of albumin.

The dialyzed globulins could not be completely redissolved in 5 per cent sodium chloride solution. Coagulation tests made on the soluble portion indicated that both globulins were denatured in about the same proportion. Both globulins are completely precipitated when their solutions are strongly acidified with acetic acid.

A mixture of the globulins (prepared either by dialysis or by acidification) and 5 per cent sodium chloride solution gave an acid reaction to litmus. Careful neutralization with sodium hydroxide, however, caused the suspended portion of the proteins to dissolve completely.

Preparations of the globulins for analysis were therefore made as follows: Sodium chloride extracts of the flour were dialyzed until the dialysate gave no test for chlorides. The precipitated globulins were suspended in 5 per cent sodium chloride solution, and the mixture made neutral to litmus with sodium hydroxide. From the filtered solution the two globulins were separated by fractional heat coagulation. The coagula were thoroughly washed with boiling water, and finally dried with alcohol and ether in the usual way. The fractions coagulating at 74 and 90°C. amounted respectively to 0.09 and 0.07 per cent of the flour. On account of the small yields obtained, several preparations of the globulins

TABLE II.

*Elementary Composition of Proteins Isolated from White Rice.*  
Percentages calculated on a moisture- and ash-free basis.

	Globulin coagulable at 74°C.	Globulin coagulable at 90°C.	Glutelin.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	52.83	49.15	51.92
H.....	6.77	7.86	6.39
N.....	16.31	17.94	16.81
S.....	0.98	1.45	0.94
Ash.....	0.29	1.64	0.75
Moisture.....	4.83	4.91	5.49

were combined to form one sample of each protein for analyses. The elementary composition of these globulins is given in Table II.

*Analyses of Globulins by the Van Slyke Method.*

Samples of 3 gm. of each of the globulins were hydrolyzed by boiling for 40 hours with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by the ether-amyl alcohol method. The results of the analyses are given in Tables III to VI.

*Glutelins.*

A quantity (500 gm.) of the rice flour was extracted with 5 per cent sodium chloride solution until practically all soluble material

TABLE III.

*Distribution of Nitrogen in the Globulin of White Rice Coagulable at 74°C. as Determined by the Van Slyke Method.\**

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N.....	0.0320	0.0322	6.89	6.94	6.91
Humin " adsorbed by lime...	0.0066	0.0065	1.42	1.40	1.41
" " in ether-amyl alcohol extract.....	0.0004	0.0006	0.09	0.13	0.11
Cystine N.....	0.0074	0.0075	1.59	1.62	1.61
Arginine N.....	0.0720	0.0717	15.51	15.44	15.48
Histidine N.....	0.0177	0.0195	3.81	4.20	4.01
Lysine N. ....	0.0394	0.0385	8.49	8.29	8.39
Amino " of filtrate.....	0.2634	0.2628	56.74	56.61	56.67
Non-amino N of filtrate.....	0.0249	0.0262	5.36	5.64	5.50
Totals.....	0.4638	0.4655	99.90	100.27	100.09

\* The two samples of globulin analyzed weighed each 2.8464 gm. (ash- and moisture-free) and contained 0.4642 gm. of nitrogen.

TABLE IV.

*Distribution of Nitrogen in the Globulin of White Rice Coagulable at 90°C. as Determined by the Van Slyke Method.\**

	I	II†	I	II†	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N.....	0.0392	(0.0392)	7.79	(7.79)	7.79
Humin " adsorbed by lime...	0.0039	(0.0039)	0.78	(0.78)	0.78
" " in ether-amyl alcohol extract.....	0.0004	(0.0004)	0.08	(0.08)	0.08
Cystine N.....	0.0096	0.0093	1.91	1.85	1.88
Arginine N.....	0.1366	0.1373	27.16	27.30	27.23
Histidine N.....	0.0227	0.0230	4.51	4.57	4.54
Lysine N. ....	0.0195	0.0195	3.88	3.88	3.88
Amino " of filtrate.....	0.2508	0.2515	49.87	50.01	49.94
Non-amino N of filtrate.....	0.0191	0.0192	3.81	3.81	3.81
Totals.....	0.5019	0.5033	99.79	100.07	99.93

\* The sample of globulin analyzed weighed 2.8035 gm. (ash- and moisture-free) and contained 0.5029 gm. of nitrogen.

† The figures in parentheses do not represent duplicate results but are carried over from the foregoing columns so as to be included in the summation.

was removed. The residue was then exhaustively extracted with tenth normal sodium hydroxide solution, and the extract made

TABLE V.

*Distribution of Nitrogen in the Globulins of White Rice as Calculated from the Van Slyke Analyses in Terms of Percentage of Proteins.*

Average of duplicate analyses.

Nitrogen.	Globulin coagulable at 74°C.*			Globulin coagulable at 90°C.†		
	I	II	Average.	I	II	Average.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Amide.....	1.12	1.13	1.13	1.40	1.40	1.40
Humins.....	0.25	0.25	0.25	0.15	0.15	0.15
Basic.....	4.80	4.82	4.81	6.72	6.75	6.74
Non-basic.....	10.13	10.15	10.14	9.63	9.66	9.64
Total.....	16.30	16.35	16.33	17.90	17.96	17.93

\* Nitrogen content 16.31 per cent.

† Nitrogen content 17.94 per cent.

TABLE VI.

*Some Amino Acids of the Globulins of White Rice.\**

Expressed as percentages of the ash- and moisture-free proteins.

Amino acids.	Globulin coagulable at 74°C.	Globulin coagulable at 90°C.	Glutelin
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Cystine.....	2.25	2.89	
“ †.....	2.11	2.87	1.10
Arginine.....	7.85	15.18	
Histidine.....	2.42	3.01	
Lysine.....	7.14	3.63	
Tryptophane.†.....	2.69	2.32	2.54
Tyrosine.†.....	5.60	7.53	5.33

\* The percentages of amino acids given in Table VI, with the exceptions noted, were calculated from the results obtained by the Van Slyke method of analysis.

† Determined colorimetrically.

slightly acid with acetic acid. The resulting precipitate was separated by centrifugation, and redissolved in 0.2 per cent sodium hydroxide. After filtering off a small amount of insoluble material

the glutelin was again reprecipitated as before, washed, and dried in the usual way. The yield was 12 gm., equivalent to 2.4 per cent of the flour used. The elementary composition of the glutelin thus prepared is given in Table II.

*Determinations of Tyrosine, Tryptophane, and Cystine.*

Tryptophane was determined in the globulins and the glutelin by the colorimetric method of May and Rose (8) with some slight modifications (9). Tyrosine and cystine were determined colorimetrically by the method of Folin and Looney (10). The results are given in Table VI, together with the percentages of the basic amino acids as calculated from the results of the Van Slyke analyses.

*Commercial Rice Bran.*

The material used for these experiments was the milling by-products obtained during the preparation of the white rice used in the preceding experiments. When removing the branny seed coats from rice, there is also removed the germ or embryo. Varying quantities of fragments of the endosperm are also usually found admixed with this by-product. On account of the finely divided condition of both the bran and the embryo we were unable to effect a separation of one from the other.

The larger particles of the endosperm were removed by sifting the material through a 40 mesh sieve. The sifted product was then extracted with ether to remove fatty matter which quickly becomes rancid. All of the experiments hereafter described were made on this prepared bran meal, the composition of which and also that of the original material are given in Table I.

Successive exhaustive extractions of the bran meal with 4 per cent sodium chloride solution, boiling 70 per cent alcohol, and 0.5 per cent aqueous sodium hydroxide solution removed respectively 1.84, 0.13, and 0.26 per cent of nitrogen. The total nitrogen extracted was equivalent to 90.1 per cent of the total calculated ( $N \times 6.25$ ) protein in the meal.

Dilution of the sodium chloride extract of the meal with 10 volumes of water gave no precipitate unless slightly acidified with acetic acid. Acidification without dilution, however, caused precipitation of the globulins. The filtrate from the protein pre-



precipitated by dilution and acidification yielded no coagulum when heated to 65°C. and but a very slight coagulum at 75°C. On the other hand, the filtrate from the protein precipitated by acidification without dilution yielded coagula at 50, 64–68, and 72–84°C. When this filtrate was chilled with ice, a substance separated which redissolved on warming to room temperature.

Acidification and chilling, or acidification and dilution with water, caused the precipitation of the fractions coagulating at 50 and 68°C. On account of the small quantity of this precipitate available, we were unable to learn anything more about it.

TABLE VII.

*Elementary Composition of Proteins Isolated from Prepared Rice Bran.\**

	Albumin fraction.	Globulin preparations.						
		I	II	III†	IV	V	VI	Average.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C.....	49.92	48.38	49.94	49.81	48.48	49.28	48.28	49.03
H.....	7.16	7.03	7.03	6.84	7.11	7.23	7.21	7.08
N.....	16.28	17.33	17.46	17.09	17.26	17.32	17.02	17.25
S.....	1.19	1.04	1.08	1.04	1.14	1.17	1.00	1.08
Moisture.....	5.74	3.55	10.28	8.46	12.19	5.12	9.26	
Ash.....	6.04	1.05	0.91	0.11	0.62	0.40	1.25	

\* Average of duplicate analyses. Percentages calculated on the ash- and moisture-free proteins.

† First precipitated by ammonium sulfate and then reprecipitated by dialysis.

No attempt was made to separate the different globulins. In view of the fact that the meal we were working with was a mixture of bran and embryo, such a separation would have but little significance.

Six preparations of the mixed globulins were made, all but one of which were obtained by dialysis of sodium chloride extracts of the meal. Preparation III was first precipitated from the sodium chloride extract by means of ammonium sulfate. The precipitate was then redissolved by addition of water, the solution filtered, and the globulin reprecipitated by dialysis. Heat coagulation

tests made on 4 per cent sodium chloride solutions of these dialysis precipitates indicated the presence of three globulins coagulating at 75, 84, and 95°C. The yields of the total globulins obtained amounted to 2.88 per cent of the meal. The elementary composition of the globulin preparations is given in Table VII. Colorimetric determinations of cystine, tyrosine, and tryptophane made on a sample of the globulin preparation showed it to contain the following percentages: cystine 2.23, tyrosine 6.20, and tryptophane 3.28.

Coagulation tests made on the dialysates from which the globulins had been removed indicated the presence of two albumins coagulating at 65 and 70°C. The coagula were thoroughly washed with hot water and dried in the usual way. The yield amounted to 0.13 per cent of the meal. On account of the small quantities obtained, several preparations of this fraction were united to form one sample, the composition of which is given in Table VII. Colorimetric determinations upon this product gave the following percentages: cystine 3.58, tyrosine 5.08, and tryptophane 3.28. In view of the low yields and of the peculiar properties already referred to of some of the fractions separating from the saline extract of the meal at low coagulating temperatures, we are unable to state whether or not the proteins of this fraction really represent true albumins originally present in the flour.

Extraction with boiling 70 per cent alcohol of either the residue remaining after extraction of the flour with sodium chloride solution, or of the unextracted flour, removed but a small quantity of nitrogen. The alcoholic extract was concentrated to a sirup, which was treated with several volumes of absolute alcohol. A substance was precipitated which was low in nitrogen and high in ash. Although by repeated washing with hot water the percentage of nitrogen was materially raised, due to the removal of inorganic salts, we were, however, unable to obtain a product that contained more than 11.2 per cent of nitrogen. It contained 11.59 per cent of ash, which had the following percentage composition: magnesium as MgO 4.53, potassium and sodium as chlorides 56.75, phosphorus as  $P_2O_5$  6.20, calcium a trace.

We were unable to isolate any protein having the properties of a glutelin.

Grateful acknowledgment is hereby made to Dr. C.E. Chambliss, Agronomist in Charge of Rice Investigations of the Bureau of Plant Industry, through whose kind cooperation the rice used in this investigation was obtained.

#### SUMMARY.

Two globulins have been isolated from the endosperm of rice, *Oryza sativa*. These proteins differ both in the temperatures at which they coagulate (74 and 90°C.), and also in their elementary composition and distribution of nitrogen. The presence of albumin could not be detected.

The globulin, albumin, and alcohol-soluble fractions obtained from commercial rice bran were also studied.

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## STUDIES ON GLUTELINS.

### II. THE GLUTELIN OF RICE (*ORYZA SATIVA*).

By D. BREESE JONES AND FRANK A. CSONKA.

*(From the Protein and Nutrition Division, Bureau of Chemistry and Soils,  
United States Department of Agriculture, Washington.)*

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A new method for the preparation of glutelins was recently described in the first article of this series (1). This method is based on the fact that glutelins can be precipitated from dilute alkaline solutions by the addition of very small amounts of ammonium sulfate. By applying this method of separation to the proteins of wheat endosperm, two glutelin fractions were isolated which differed not only in the amounts of ammonium sulfate required for their precipitation, but also in their chemical composition.

In connection with the work done in this laboratory during recent years, on the proteins of different seeds, it has been shown that most of them contain at least two globulins designated as  $\alpha$ - and  $\beta$ -globulins, which differ not only in their physical and chemical properties but also in their immunological (2) reactions. These results suggest that not only the globulin fraction obtained from a given source may consist of more than one globulin, but that the other classes of protein fractions as albumins, prolamins, and glutelins may also in many cases be capable of further fractionation. The results that we have obtained with wheat glutelin give further support to this view. The general method heretofore used for the preparation of glutelins does not readily lend itself to the pursuance of this phase of the study of glutelins. The method referred to for the isolation of glutelins, depending on their precipitation with ammonium sulfate, offers the same means for the possible fractional precipitation of different glutelins as were used in the case of the globulins. It is therefore a matter of interest to examine the glutelin fractions of other cereals than wheat to see whether they contain more than one glutelin.

The method of ammonium sulfate precipitation has now been applied to the study of rice glutelin. Rice glutelin, which was named oryzenin by Rosenheim and Kajiura (3), represents the greater portion of the proteins of the rice endosperm. In order to differentiate our product from the preparations of others made by a different method, we shall refer to it as rice glutelin rather than by the specific name oryzenin. A multiplicity of specific names is more or less confusing, and they do not show to which class the protein belongs. There are other proteins besides glutelin in rice endosperm (4, 5) to which the name oryzenin could be equally appropriately applied.

TABLE I.  
*Composition of Rice Glutelin.\**

	Authors.	Jones and Gersdorff.	Osborne, Van Slyke, Leavenworth, and Vinograd.	Kondo and Hayaashi.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	52.58	51.92		
H.....	6.42	6.39		
N.....	17.57	16.81	16.68	16.91
S.....	1.65	0.94		
P.....	Trace.			
Ash.†.....	0.384	0.74	0.78	

\* Percentages, except as otherwise indicated, were calculated on an ash- and moisture-free basis.

† Calculated on a moisture-free basis.

By using the ammonium sulfate method of preparation we have been able to find only one glutelin in polished rice. The elementary composition of this product is shown in Table I. It will be noted that the percentage of nitrogen found is higher than that reported for rice glutelin prepared by acid precipitation (4, 6, 7). In Tables II and III are given the results of the distribution of nitrogen by the Van Slyke method. Compared with the figures given by others for rice glutelin, which for the sake of comparison are also included in the tables, the chief differences lie in the percentages of cystine and histidine nitrogen. The figure for cystine is higher than that found by Osborne, Van Slyke, Leavenworth, and Vinograd, but is far from approaching the extraordinarily high

amount reported by Kondo and Hayashi (7). The isoelectric point of the rice glutelin as determined by the method we have recently described (8) was found to be at pH 6.45. It should be noted that the solubility curve has a break where the use of phosphate buffer commences. The lowest solubility, however, was

TABLE II.  
*Distribution of Nitrogen in Rice Glutelin.*

Nitrogen.	Authors.	Osborne, Van Slyke, Leavenworth, and Vinograd.	Kondo and Hayashi.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Amide.....	10.96	11.33	10.70
Humin.....	1.06	1.59	0.86
Cystine.....	1.56	0.88	6.18
Arginine.....	20.38	17.69	16.36
Histidine.....	3.68	5.39	5.85
Lysine.....	5.16	4.90	7.35
Amino N of filtrate.....	54.33	52.98	
Non-amino N of filtrate.....	2.30	5.28	

TABLE III.  
*Rice Glutelin.*

Gm. of amino acids per 100 gm. of protein.\*

	Authors.	Osborne, Van Slyke, Leavenworth, and Vinograd.	Jones and Gersdorff.	Kondo and Hayashi.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Arginine.....	11.13	9.15		8.40
Histidine.....	2.39	3.32		3.65
Lysine.....	4.73	4.26		6.48
Cystine.....	2.35	1.26	1.10†	8.95

\* Calculated from the results of analyses by the Van Slyke method, with the exception noted.

† Determined colorimetrically by the method of Folin and Looney (9).

observed at pH 6.45. The amount of prolamin present in rice endosperm is so small that we did not think it necessary to extract the rice flour first with dilute alcohol. The precipitated glutelin, however, was washed several times with 60 per cent alcohol previous to dehydration with absolute alcohol and ether.

*Preparation of Rice Glutelin.*

1 kilo of rice flour prepared in the laboratory from polished rice was stirred with 4 liters of 0.2 per cent sodium hydroxide for 6 hours. After allowing the mixture to stand overnight, the supernatant liquid was siphoned off and filtered by suction through a mat of paper pulp. A preliminary test made on the clear filtrate showed that the glutelin was precipitated from its alkaline solution when enough of a saturated ammonium sulfate solution was added to make the final volume 0.023 saturated with that salt. The main portion of the clear filtrate was made 0.03 saturated with ammonium sulfate, and the resulting precipitate allowed to settle. The supernatant liquid containing the other proteins of the rice flour was discarded and the sediment centrifuged and washed several times with 0.03 saturated solution of ammonium sulfate. The glutelin was redissolved in 0.2 per cent sodium hydroxide, filtered, and reprecipitated with ammonium sulfate and washed as before. The product was then washed, first with distilled water slightly acidified with HCl (pH 6.4), and then three times with 60 per cent alcohol followed by two washings with 95 per cent alcohol. The product was finally dried in the usual way with absolute alcohol and ether. The yield of the final product was 15 gm. This, of course, does not represent the actual amount of glutelin present in polished rice, since considerable losses were involved during the precipitation and purification of the protein.

## SUMMARY.

The method previously used whereby it was shown that wheat endosperm contains two differentutelins has been now applied to the proteins of polished rice. The glutelin was precipitated from a 0.2 per cent sodium hydroxide extract by making the latter 0.03 saturated with ammonium sulfate. In contrast with wheat endosperm, polished rice contains only one glutelin. Percentages are given showing the elementary composition and the distribution of nitrogen in the rice glutelin. Its isoelectric point was found to be at pH 6.45.

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## THE IRON CONTENT OF ANIMAL TISSUES.\*

By C. A. ELVEHJEM AND W. H. PETERSON.

*(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)*

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In a recent paper (1) we have shown that the Thomson method cannot be used to determine the iron content of biological material high in phosphorus because the presence of excess phosphate causes a fading of the ferric thiocyanate. This difficulty was overcome by removing the phosphorus, according to our modified method, before developing the color. The results obtained by the use of this method for the determination of the iron content of milk and other food materials have been so successful that its application to animal tissues seemed advisable.

No detailed study of the iron content of the various animal tissues has been made probably because of the lack of suitable methods. Information of this sort as well as available methods for making such an analysis is certainly necessary at this time when the effect of diet upon the hemoglobin-building power of the animal is so much under consideration. The need is especially evident since some of the workers (2, 3) so strongly stress the value of certain animal tissues such as liver, and muscle in a lesser degree, in hemoglobin regeneration.

While our work was in progress Forbes and Swift (4) published a paper in which they point out the lack of reliable information on the iron content of meats and give analyses of a number of the common meat products and the principal organs of cattle. They did not include the entire list of beef organs. Nor did they give the complete method of iron determination. They state that the iron was determined by titration with standard  $\text{KMnO}_4$  solution, but do not say how the iron was reduced before titrating.

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The reduction of the iron is the most difficult part of the method and is the procedure which has been most subject to unfavorable criticism.

*Methods of Analysis.*—In all our work we have used either the Thomson (1) method or the modified method depending upon whether or not phosphate interference was encountered. Since the modified method is the longer of the two, it is advantageous to use the Thomson method whenever possible. In order to determine whether the modified method was necessary all samples were first analyzed by the Thomson method. The determinations were run in triplicate and 1 cc. of standard iron solution, equivalent to 0.1 mg. of Fe, was added to the filtrate of one of the triplicates after the ash had been taken up in concentrated HCl, diluted, and filtered from the insoluble residue. The three samples were then carried through together and the average of the amount of iron found in Samples 1 and 2 subtracted from that of Sample 3 to determine the recovery of the added iron. From this figure the percentage of iron recovered was calculated. An error of 5 per cent in either direction was allowed. Thus if a recovery between 95 to 105 per cent was obtained the determination was regarded as satisfactory. Where the recovery was not complete a definite fading of the color was generally observed. If a satisfactory recovery of added iron was obtained the result from the Thomson method was taken and the percentage of iron in the sample calculated. If the recovery was not complete the analysis was repeated by use of the modified method. The practice of adding 0.1 mg. of Fe was also used in this method to check the accuracy of the determination.

#### *Analysis of Various Tissues.*

*Beef.*—The first series of analyses made was on the various tissues of a beef animal. The samples were obtained directly from a local packing house, brought to the laboratory, and prepared immediately. Each sample was carefully washed, cut into small portions, placed in large porcelain evaporating dishes and dried at 65°C. and finally at 98°C. During the process of drying, each preparation was closely watched and intermittently stirred to prevent decomposition. After they were completely dry they were ground in a large mortar instead of a mill so that there was

TABLE I.  
*Iron Content of Beef Tissues.*

Kind of tissue.	Method used.	Weight of sample.	Iron in sample.	Iron in sample + 0.1 mg. Fe.	Recovery of added iron.	Iron in sample, dry basis.
		gm.	mg.	mg.	per cent	per cent
Bone marrow.....	Thomson.	10.0	0.105	0.203	98	0.0010
Brain.....	"	1.0	0.136	0.232	96	0.0134
Hair.....	"	0.3	0.274	0.378	104	0.0920
Heart.....	"	1.0	0.235	0.337	102	0.0236
Hide.....	Modified.	1.0	0.175	0.272	97	0.0173
Intestine.....	"	1.0	0.184	0.279	95	0.0181
Kidney.....	"	0.5	0.146	0.151	105	0.0292
Liver.....	"	0.5	0.147	0.143	96	0.0294
Lung.....	Thomson.	1.0	0.624	0.719	95	0.0621
Muscle, round steak.....	Modified.	1.0	0.163	0.266	103	0.0164
"    T-bone    ".....	"	1.0	0.143	0.240	97	0.0141
Pancreas.....	"	1.0	0.300	0.398	98	0.0299
Spleen.....	Thomson.	0.5	0.115	0.110	95	0.0383

TABLE II.  
*Dry Matter and Iron Content of Fresh Beef Tissues.*

Kind of tissue.	Dry matter in tissue.	Iron in dry matter.	Iron in fresh tissue.	Iron in fresh tissue. (Forbes and Swift.)
	per cent	per cent	per cent	per cent
Bone marrow.....	91.5	0.0010	0.0009	
Brain.....	17.4	0.0134	0.0023	0.0053
Hair.....	88.2	0.0920	0.0811	
Heart.....	20.6	0.0236	0.0048	0.0044
Hide.....	27.0	0.0173	0.0047	
Intestine.....	18.9	0.0181	0.0034	
Kidney.....	18.9	0.0292	0.0055	0.0188
Liver.....	28.4	0.0294	0.0083	0.0082
Lung.....	19.7	0.0621	0.0122	
Muscle, round steak.....	24.9	0.0164	0.0041	0.0025
"    T-bone    ".....	26.0	0.0141	0.0037	0.0025
Pancreas.....	20.0	0.0299	0.0060	
Spleen.....	23.2	0.0383	0.0089	0.0138

no chance for contamination with any iron. The samples were then used directly for the iron determination. The results ob-

TABLE III.

*Iron Content of Spleen, Liver, and Kidney from Different Animals.*

Kind of tissue.	No. of tissues analyzed.	Dry matter in tissue.	Iron in dry matter.	Iron in fresh tissue.	Variation.	
					Mini-mum.	Maxi-mum.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Spleen, beef.....	20	22.9	0.0397	0.0091	0.0070	0.0118
“ calf.....	19	21.7	0.1176	0.0255	0.0162	0.0384
“ hog.....	1	22.1	0.1330	0.0294		
Liver, beef.....	4	28.4	0.0294	0.0083	0.0081	0.0085
“ calf.....	4	26.8	0.0203	0.0054	0.0046	0.0060
“ hog.....	4	31.3	0.0800	0.0250	0.0132	0.0391
Kidney, beef.....	5	20.4	0.0280	0.0057	0.0041	0.0082
“ hog.....	4	20.9	0.0284	0.0059	0.0047	0.0075

TABLE IV.

*Iron Content of Tissues from Rabbit 1.*

Kind of tissue.	Method used.	Weight of sample.	Iron in sample.	Iron in sample + 0.1 mg. Fe.	Recov-ery of iron.	Iron in dry material.
		<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
Bone, humerus.....	Modified.	1.0	0.191	0.292	101	0.0192
“ femur and tibia.....	“	1.0	0.177	0.278	101	0.0177
Brain.....	Thomson.	0.077	0.038	0.137	99	0.0482
Heart.....	“	0.216	0.122	0.227	105	0.0573
Hide and hair.....	“	1.0	0.119	0.222	103	0.0121
Intestine.....	Modified.	0.564	0.091	0.185	94	0.0153
Kidney.....	Thomson.	0.298	0.098	0.196	98	0.0324
Liver.....	“	0.5	0.283	0.381	98	0.0564
Lung.....	“	0.188	0.073	0.171	98	0.0376
Muscle.....	Modified.	2.0	0.180	0.277	97	0.0089
Spleen.....	Thomson.	0.040	0.074	0.175	101	0.1867
Stomach.....	Modified.	0.342	0.132	0.235	103	0.0390

tained upon analysis of the various beef tissues are given in Table I.

The moisture content of the different tissues was determined by taking two 5 gm. samples while the tissues were being prepared

for the drying process. The samples were dried to constant weight at 98°C., the loss of weight determined, and percentage of dry matter calculated.

Table II gives the percentage of dry matter in the tissues and the percentage of iron calculated on the fresh tissue. The results of Forbes and Swift are also given where possible for comparison with our results.

Since the iron content of the liver, spleen, and kidney is receiving special attention at the present time, additional analyses

TABLE V.  
*Iron Content of Tissues from Rabbit 2.*

Kind of tissue.	Method used.	Weight of sample.	Iron in sample.	Iron in sample + 0.1 mg. Fe.	Recovery of iron.	Iron in dry material.
		gm.	mg.	mg.	per cent	per cent
Bone, humerus.....	Modified.	0.653	0.173	0.274	101	0.0264
“ femur and tibia.....	“	1.048	0.202	0.303	101	0.0192
Brain.....	Thomson.	0.217	0.100	0.206	106	0.0473
Heart.....	“	0.175	0.065	0.160	95	0.0358
Hide and hair.....	“	1.100	0.111	0.206	95	0.0099
Intestine.....	Modified.	0.657	0.123	0.227	104	0.0190
Kidney.....	Thomson.	0.334	0.083	0.187	104	0.0254
Liver.....	“	0.402	0.125	0.222	97	0.0307
Lung.....	“	0.279	0.079	0.185	106	0.0297
Muscle.....	Modified.	1.065	0.104	0.202	98	0.0096
Spleen.....	Thomson.	0.087	0.114	0.211	97	0.1284
Stomach.....	Modified.	0.458	0.103	0.206	103	0.0228

were made on these tissues taken from a large number of animals. The results of these analyses are given in Table III.

*Rabbit.*—The second series of analyses was made on the various tissues of rabbits. Rabbits were used so that all dissection could be done in the laboratory where there was no danger of iron contamination and so that all the tissues could be obtained from the same animal. The rabbits were killed by decapitation and thoroughly bled to prevent accumulation of blood in any of the organs. After careful removal, the tissues were dried and the moisture content determined exactly as in the case of the beef tissues. In many cases the different samples were so small that

the entire organ was used for the iron determination. Analyses were made on the tissues of two rabbits; those from Animal 1 are given in Table IV and those from Animal 2 in Table V.

In Table VI are tabulated the percentage of dry matter in the different tissues of the rabbits and the iron content of the fresh tissues calculated from the average percentage of iron in the dried tissues.

TABLE VI.

*Dry Matter and Iron Content of Rabbit Tissues (Average of Figures for Rabbits 1 and 2).*

Kind of tissue.	Dry matter in tissue.	Iron in dry matter.	Iron in fresh tissue.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Bone, humerus.....	65.1	0.0228	0.0148
“ femur and tibia.....	68.3	0.0185	0.0126
Brain.....	22.8	0.0477	0.0109
Heart.....	20.9	0.0464	0.0099
Hide.....	35.7	0.0110	0.0039
Intestine.....	21.9	0.0171	0.0037
Kidney.....	21.7	0.0289	0.0063
Liver.....	30.4	0.0435	0.0132
Lung.....	19.8	0.0338	0.0067
Muscle.....	21.6	0.0092	0.0020
Spleen.....	21.4	0.1565	0.0336
Stomach.....	22.1	0.0307	0.0068

## DISCUSSION.

Upon studying the methods of analysis used for the different tissues, it is found that the modified method is required for seven of the beef tissues and five of the rabbit tissues. Although one readily notices that tissues relatively low in iron and high in phosphorus are the samples which necessitate the removal of the phosphorus, it is impossible to generalize and give any definite percentage of phosphorus above which the Thomson method cannot be used. The best and most accurate procedure to adopt for determining whether the Thomson method is suitable is to determine whether or not there is a complete recovery of the added iron. In some cases the use of the modified method may be avoided by reducing the weight of the sample taken. For example, we were able to do this in the case of the liver and kidney

of the rabbit. The use of a 1 gm. sample produced a definite fading but the use of 0.5 gm. gave no fading. Analyses were made by both methods on the liver and kidney of Rabbits 1 and 2 and excellent checks were obtained. This could not be done, however, in the case of the beef tissues. According to Kossel (5) the liver and kidney of the rabbit are considerably lower in phosphorus than the same tissues from cattle.

In general we may say that the iron content of the internal organs is higher than that of other tissues. The iron content of muscle was found to be quite low. Although our figure for beef muscle (0.0039 per cent average) is somewhat higher than that found by Forbes and Swift (0.0025 per cent), it compares very well with those of Bunge (6) (0.0035 per cent) and Stockman (7) (0.0039 per cent). The iron content of bone marrow was found to be very low, and that of the brain, intestine, and hide was also quite low. The lung is very high in iron. Hair was found to be rich in this element.

Of the four internal organs, kidney, liver, pancreas, and spleen, the spleen was found to be the highest in iron, with the liver, pancreas, and kidney following in order. Our figures for spleen and liver check well with those of Forbes and Swift, but our figures for kidney are considerably lower. The lower figure is verified, however, by the average of a number of analyses shown in Table III.

The figures obtained for the rabbit tissues check fairly well with those of the beef tissues. There are some variations, which is to be expected, but most of the figures are of the same general order of magnitude. The iron content of raw bone, as shown by the analyses of the humeri and femurs from the rabbits, is quite high and is comparable to that of liver. That there is considerable variation in the iron content in the same kinds of tissues taken from different animals is shown by comparing the figures for Rabbits 1 and 2.

A representative figure for tissues from any specific animal may be obtained by increasing the number of tissues used for the analysis. Our analysis of beef spleen and calf spleen, in Table III, demonstrated this very well. For example, it was found that the iron content of beef spleen, dry basis, averaged 0.0402 per cent Fe when five samples were analyzed separately,



and the iron content of a composite sample of fifteen spleens was found to be 0.0397 per cent.

The data presented in Table III are of great practical importance when these tissues are used in the treatment of anemia. Calf spleen is over twice as rich in iron as beef spleen. This may be explained by the fact that these young animals may still retain considerable of the iron supply present at birth. The spleens were obtained from calves from 4 to 6 weeks old. Calf liver is somewhat lower in iron than beef liver, but hog liver is very much higher. The fact that calf liver is lower in iron than beef liver is difficult to explain unless we suppose that the store of iron in the liver at birth is exhausted. Since calves of 4 to 6 weeks of age are on a diet consisting mostly of milk, the iron supply in the liver may be somewhat depleted. The tabulation of the maximum and minimum percentages of iron obtained for the different tissues gives an idea of the variation in the iron content in different samples.

From the standpoint of iron content hog liver is the best type of liver to use in the cure and prevention of anemias. Of course variations in the individual tissues must be recognized, but by using livers from this animal the total average is retained at a high level.

In view of the fact that calf liver is lower in iron than the livers of other animals analyzed, it becomes a question whether in infant feeding the livers of animals other than that of the calf should not be considered.

Another very interesting fact noted in regard to material used in infant feeding is the small amount of iron in beef juice. Beef juice prepared in the same way as it is for infant feeding is found to contain only 0.0029 per cent Fe. The percentage of the iron in the beef juice was only 11.8 per cent of that present in the original beef from which the juice was expressed. These results suggest the need of further study of some of the methods used at the present time to furnish infants additional iron.

In all work dealing with the iron content of animal tissues the criticism may always be made that the tissues are contaminated with blood which increases the iron content. We believe that this possibility has been reduced to the minimum because every care has been used to prevent any contamination with blood.

However in the case of edible organs this criticism is without basis, for any blood retained in the organ must be regarded from a food point of view as part of that organ.

#### SUMMARY.

1. Data are presented to show that by the use of either the Thomson method or the modified method suggested by Elvehjem and Hart, the iron content of animal tissues may be determined accurately.

2. A means of determining whether the modified method is necessary for the analysis has been devised which consists of measuring the recovery of added iron.

3. Analyses are given of thirteen beef tissues and twelve rabbit tissues.

4. Tissues such as bone marrow, hide, intestines, heart, and muscle are low in iron. Tissues such as lung, spleen, liver, and kidney are relatively high in iron.

5. A number of analyses of spleen, liver, and kidney taken from different animals are given. These results may aid in the selection of liver high in iron for use in the treatment of anemias.

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# A DENSIMETER FOR THE RAPID DETERMINATION OF THE SPECIFIC GRAVITY OF SMALL QUANTITIES OF LIQUIDS AND SOLIDS.

By P. LECOMTE DU NOÛY.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

## PLATE 1.

(Received for publication, May 9, 1927.)

For a long time there has been a need for an instrument whereby the specific gravity of 1 cc. of liquid could be ascertained to the third decimal place very rapidly. Such an apparatus has been devised by improving the model which was demonstrated at the meeting of the Federation of American Societies for Experimental Biology in Baltimore, 1918.<sup>1</sup> The principle adopted is exactly the same as that used in our tensiometer; namely, a torsion balance fitted with a dial and a vernier. The instrument is likewise direct reading, and accurate measurements are made with 1 cc. of liquid in a few seconds.

### *Description and Adjustment.*

#### *I. Liquids.*

The main difference between the tensiometer and the densimeter is that in the latter the weight of the bob is balanced by a counterweight supported by an extension of the arm, so that the twisting of the wire only takes care of the final accurate adjustment of the zero (Fig. 1). The zero indicator consists of two fine pointers facing each other, one being fixed to the frame and one to the arm. A perfect zero balance can thus be obtained without parallax error. The range of the dial of the instrument, which is graduated on all its periphery, is between 0 and 2.800, but the range of utili-

<sup>1</sup> du Noüy, P. L., *Am. J. Physiol.*, 1919, xlix, 123.

zation is greater, since it is only limited by the specific gravity of the bob. The second decimal place is given on the dial by divisions about 1 mm. apart, and the third decimal is read on the vernier.

The bob for liquids having a density not above 2.500 is made of ordinary glass. Heavy flint may be used when higher densities are being measured (up to 4.500), and alloys if still higher values are sought. Its dimensions are: length, including hook, 31 mm.; diameter, 6.3 mm. The suspension is made out of platinum wire, 0.075 mm. in diameter, so as to reduce the error due to capillary action. This action is negligible as it amounts to about 0.00012 gm. A wire twice as thick could therefore be used without introducing a measurable error. The liquid to be tested is contained in a small glass cylinder, the inside diameter of which is only 1 mm. larger than that of the bob. The temperature is controlled by keeping this cylinder in a water bath or, if more than 1 cc. of liquid is available, by using a special vessel in the shape of twin cylinders, in one of which stands the thermometer. In this case, 2 to 2.5 cc. of liquid are required.

The adjustment is made in the following way: the torsion wire is loosened so as to be submitted to no torque at all. While it is loose, the vernier is set on the 1.000 mark on the dial. Then the wire is clamped at both ends, the piece supporting the arm is fastened to it, and the wire is tightened. Under these conditions, the zero balance, with no bob and no counterweight, should be almost perfect. The bob and counterweight are then put in place, and the bob is immersed in the water at 4°C., or in a mixture of liquids (benzene-chloroform, for example) of specific gravity 1.000 at room temperature. The balance is then obtained roughly by means of the counterweight, and the final adjustment made by means of the torsion head, as in the tensiometer. It is obvious that, in this way, the twist of the wire is reduced to its minimum and consequently the best conditions for accuracy are fulfilled. When high specific gravities are measured, it is advisable to adjust the zero balance in a standard liquid of density greater than 1.000, by means of the counterweight, and to correct the value read on the dial accordingly.

One or two standard liquids are then chosen (for example, pure ethyl alcohol  $d = 0.789$  at 20°C., and carbon tetrachloride  $d =$

1.581 at 21° C.), and the bob is immersed. The vernier is then moved until the zero balance is obtained. If the reading on the dial differs from the standard value (checked if necessary with a hydrometer), the length of the arm supporting the bob is increased or shortened, according to the direction and magnitude of the deviation. The zero is readjusted in the liquid of density 1.000, and another reading made in alcohol or carbon tetrachloride. This procedure is repeated until a perfect check is obtained. The instrument is then calibrated and ready for use. Determinations of  $d$  take less than 5 seconds.

## II. Solids and Powders.

The determination of the specific gravity of powders is somewhat more complicated, as it is absolutely necessary to remove the air from the powder and, although the measurement itself takes only a few seconds, the preliminary technique is lengthy. In order to obtain the third decimal place, with an error not amounting to more than five units in the fourth place with 0.1 gm. of substance, the arm supporting the bob can be extended by 50 mm., so as to increase the sensitivity. By weighing exactly 0.100 gm. of powder, and by superposing another scale over that graduated for liquids, the instrument can be made direct reading. However, it seems more convenient to graduate the scale in mg., and to use a simple formula

$$(1) \quad c = \frac{m \delta}{(m - R)}$$

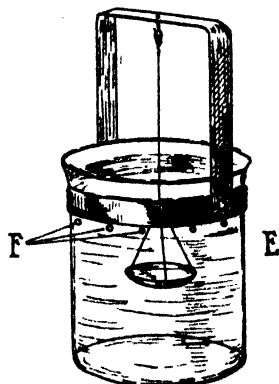
where  $m$  = weight of substance,  $R$  = reading on dial corresponding to torque, and  $\delta$  = specific gravity of liquid used. As the scale can be graduated so as to give  $m - R$  directly, the formula becomes

$$(2) \quad d = \frac{m \delta}{R}$$

The procedure is then as follows:

*Calibration of Instrument.*—A small pan suspended at the extremity of the arm is balanced by shifting the counterweight and a perfect zero balance is secured, as in the case of the bob, by the

torsion wire, the vernier being at position 1.000 on the liquids scale (namely, vertical). Then a 0.1 gm. weight is placed on the pan, and the length of the arm adjusted, until the zero balance is restored by shifting the vernier by means of the torsion knob to division 0.100 on the new scale. This being done, the zero balance is obtained with the pan immersed in the liquid to be used (liquid in which the powder does not dissolve). Then, after a given quantity of powder has been weighed to the fourth place, it is placed on the pan, and the supplementary dial is moved so as to have the zero of the vernier coincide with the weight of the substance on this dial. For example, the weight being 0.9864 gm., the dial itself is slid so that the zero of the vernier is between 0.986 and 0.987. Of course, the same thing would be done if the weight were higher than

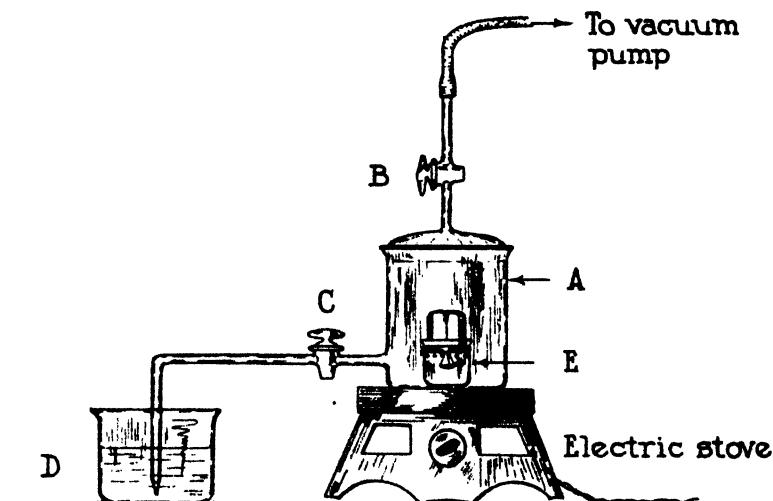


TEXT-FIG. 1. Specific gravity of powders; details of container.

0.100 gm. In this way, the zero balance does not have to be changed, and it is not necessary to weigh the sample painstakingly in order to bring it exactly to 0.100 gm.

The pan with the powder is then attached to a metal frame fixed to the glass vessel which is destined to receive the liquid (Text-fig. 1), and the whole is placed in a jar, *A*, (Text-fig. 2) fitted with two stop-cocks and a ground glass cover. This jar is then placed on a small electric stove, and the upper outlet connected with a good vacuum pump. The lower outlet ends in a capillary tube, or a tube having a tip narrowed to capillary size so as to reduce the rate of flow of the liquid passing through it. This tube dips into the container *D* which is filled with the liquid chosen for the experiment. The following technique is then used.

With stop-cock *C* closed and *B* open, the pump is started and the vessel evacuated. The vacuum, of the order of 0.01 mm. of mercury, is kept on for a few hours, in order to make sure that all the air has been removed from the powder. Then stop-cock *C* is opened very slowly and carefully, and the liquid sucked into *A*. The inside vessel *E* has small holes bored on its periphery (Text-fig. 1, *F*) and when the level of the liquid in *A* reaches these holes, the liquid, which generally has a low surface tension, flows through them and slowly fills the vessel *E*. The stop-cock *C* is closed when the level of the liquid in *A* is well above the edges of vessel *E*. With the vacuum still on, a low heat is started, so that the liquid



TEXT-FIG. 2. Apparatus for removing air from powders.

reaches the boiling point. As the pressure is low, the boiling temperature is also low. After 10 or 20 minutes, or more, according to the nature of the liquid and of the powder, the pump is stopped, air is allowed to come back into *A*, and the vessel *E* is removed by means of forceps. It is placed on the table of the densimeter, the pan is hooked to the arm of the instrument *without removing it from the liquid*, and the measurement is made. Thus, the best conditions for obtaining the specific gravity of the powder are realized. It goes without saying that when dealing with solids, even when broken into small pieces, it is not necessary to keep the vacuum on for so long a time, 10 to 15 minutes being ample.



**EXPLANATION OF PLATE 1.**

**FIG. 1. Densimeter.**

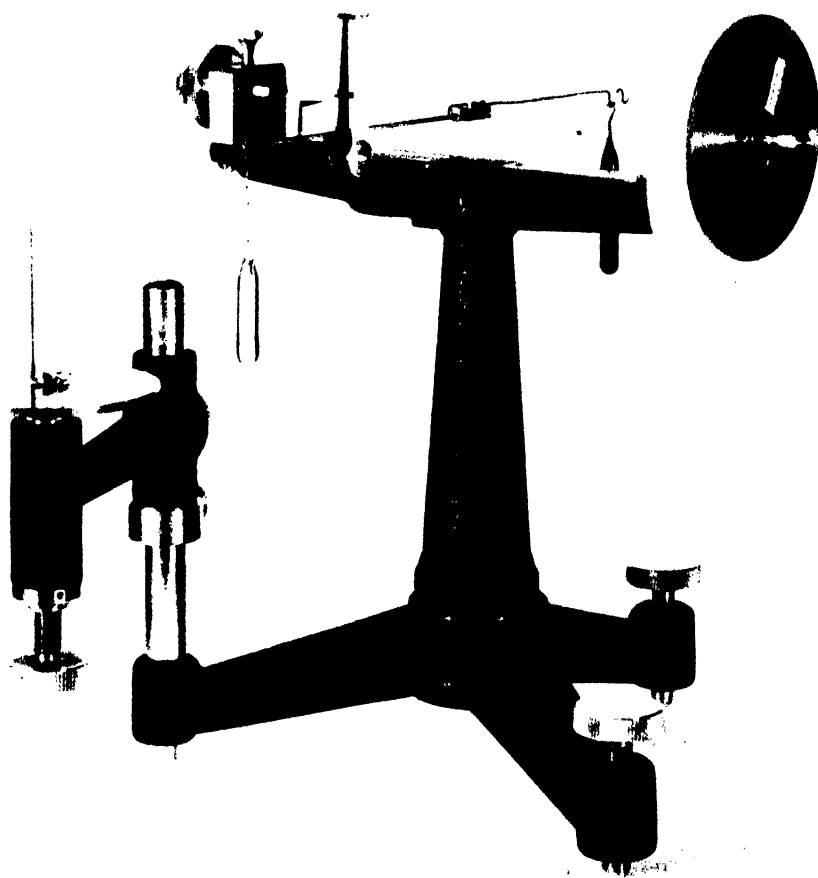


FIG. 1.

(du Nöry: Determination of specific gravity.)



# CHEMICAL AND POLARIMETRIC OBSERVATIONS ON GLUCOSE AND SALT SOLUTIONS RECOVERED FROM THIRY-VELLA LOOPS.

BY H. L. WHITE AND J. RABINOWITCH.

*(From the Department of Physiology, Washington University School of  
Medicine, St. Louis.)*

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Hewitt and Pryde (1) have reported that when a hypotonic glucose solution is allowed to stay for about 5 minutes in the small intestine of an anesthetized rabbit or rat, the dextrorotatory power is greatly reduced, but returns to normal in from 15 to 30 minutes. While no figures are given, they state that the final polarimeter readings gave concentrations of sugar which agreed with the values obtained on volumetric estimations by Benedict's method. They interpret their results as evidence of a production of  $\gamma$ -glucose on contact with living intestinal mucous membrane. Since these findings were of extreme interest to everyone concerned with the fundamentals of carbohydrate metabolism, it is not surprising that the work has been repeated. Stiven and Reid (2), using essentially the same technique as that of Hewitt and Pryde, except for a different filter, found no evidence of a mutarotation by hypotonic glucose solutions after 5 to 10 minutes stay in an intestinal loop. They obtained evidence of levorotatory material, presumably protein, in the intestinal secretion, but do not find that this mutarotates. Van Creveld (3) also failed to find any mutarotation, either with anesthetized rabbits or with a Thiry-Vella fistula dog. Hume and Denis (4) found in twelve of twenty-one satisfactory experiments on anesthetized rabbits no change in optical activity within the first 20 minutes after removal from the intestine; they found, as had Stiven and Reid, that the glucose value by titration (Shaffer-Hartmann) was higher than calculated from the observed rotation. In the other nine experiments Hume and Denis found a mutarotation, in five a small upward change,

in four a much greater downward change. While it is apparent that none of the three reports following that of Hewitt and Pryde has confirmed their findings, still it seemed worth while on the basis of the following considerations to carry out the experiments reported here.

In the first place, none of the previous experiments, with the exception of one set by Van Creveld on a Thiry-Vella fistula dog, was carried out under the most nearly possible physiological conditions. Two Thiry-Vella fistula dogs (operations by J. R.) were available in this laboratory, and in view of the extremely interesting possibilities (as the action of phlorhizin, insulin, pancreatectomy) which would present themselves in case Hewitt and Pryde's findings were confirmed on a permanent preparation, it was decided to repeat their work even in the face of the three negative reports. In the second place, we had found that 3 minutes centrifuging without any chemical treatment or filtration gave solutions sufficiently clear to read in the polarimeter if the intestinal loop was washed three times with the solution to be introduced and if the source of light was the sodium lamp designed by West (5). It was felt that this method would lessen the probabilities of a transformation of a possibly existing unstable form of glucose back to its stable form before the reading could be made. In the third place, aside from any question of mutarotation or  $\gamma$ -glucose, it seemed worth while to investigate further the finding of Stiven and Reid and of Hume and Denis that the reducing value of the intestinal returns was greater than their dextrorotatory value. By the use of Somogyi's technique (6) it was a simple matter to tell whether this discrepancy was due to an addition of non-reducing levorotatory or of non-glucose copper-reducing substance, or of both, to the intestinal contents.

In these experiments the animals lay quietly on the table while their intestinal loops were washed out three times with an approximately 2 per cent aqueous solution of glucose. They had been operated upon about 4 months before and were in excellent condition. The solutions were in every case made up the evening before the experiment and let stand in the ice box overnight to allow ample time for optical equilibrium to be established. They were introduced into the gut by gravity, first being warmed to 38°C., and were retained for 10 minutes by rubber balloons of the

type described by Gumilewski (7). One of us (J. R.) introduced and recovered the solutions, centrifuged, and handed them into the dark room. The other sat in the dark room for 10 minutes before beginning the readings. The polarimeter was a three-field instrument reading directly to 0.01 degree. The readings were all made on the same 10 cm. tube; the polarimeter scale was read by a dim, shielded light. The bright light of the sodium lamp designed by West was found to be a great advantage in this work. Individual readings are not more than 0.02° from the correct figure, *i.e.* in a rapid series of consecutive readings the lowest figure is not more than 0.04° below the highest; the average of a series is probably not more than 0.01° from the correct figure. Chemical analyses were made with Somogyi's copper reagent (8). In no case were the published tables for converting titration figures into mg. of glucose used; a series of three curves was run on known chemically pure glucose solutions and a table constructed from the average of these. The reagent was so standardized three times during the course of the work; one batch of reagent was found to vary somewhat in its titration figures; another was quite constant from one time of standardization to the next.

A few words might be given on the technique of determining true glucose and non-glucose copper-reducing substances in the intestinal returns. We are indebted to Dr. Somogyi for advice in this matter. Since the returned fluids had such a low protein content, making a very small precipitate with tungstic acid, we added 7 volumes of a 1 per cent neutral casein suspension instead of 7 volumes of distilled water, then 1 volume of 7 per cent sodium tungstate and 1 volume of 7/15 N sulfuric acid, to 1 volume of intestinal return diluted 1 to 10. To 5 cc. of this filtrate were added 5 cc. of copper reagent, the usual procedure then being carried out. This gave the glucose equivalent of the total copper-reducing substances in the solution. For the determination of the non-sugar copper-reducing substances a 20 per cent suspension of five times washed Fleischmann's yeast was made up in a 1 per cent suspension of neutral casein; to 1 volume of the solution to be analyzed an amount of suspension containing 7 volumes of water was added. A tungstic acid filtrate so obtained contains only non-sugar copper-reducing substances. We have amply confirmed Somogyi's statement that such a yeast suspension will completely

remove the glucose from solutions up to 0.3 per cent glucose, the highest we have tried. Due to the fact that we did not make up our yeast suspensions fresh every day the yeast blanks had a slightly lower titration figure (0.1 to 0.2 cc.) than the water blanks. In our experiments this was allowed for in reading the tables. In every case the titration figures of a yeast plus glucose filtrate were the same as those of the yeast blank.

Six experiments were carried out, four on one dog, two on the other. In no case was there any evidence of a mutarotation, in either direction, of the intestinal returns. The first reading was made from 5 to 7 minutes after the solution was taken from the loop and readings were continued at frequent intervals for 30 to 40 minutes. A protocol of a typical experiment follows.

*Dog B.*—June 2. Loop washed three times with glucose solution to be introduced. 12.22 p.m. introduced 50 cc. of glucose solution; 12.32 solution out, centrifuged; read at 12.39 p.m. Readings at 1 to 3 minute intervals (10 cm. tube, sodium light, zero reading  $0.10^\circ$ ) 1.09, 1.12, 1.13, 1.09, 1.10, 1.12, 1.10, 1.12, 1.13, 1.11, 1.10, 1.13, 1.10, last at 1.10 p.m. Average 1.11, minus 0.10 equals 1.01.

$$\frac{1.01 \times 100}{52.8} = 1.91 \text{ per cent glucose equivalent of returned fluid.}$$

Introduced fluid was 1.89 per cent glucose by polarimeter and 1.86 per cent by copper reduction. Returned fluid was 2.03 per cent glucose equivalent of total copper-reducing substances. Returned fluid plus yeast gave duplicate titrations of 21.80, 21.84 cc. Yeast blanks were 22.07, 22.13 cc., water blanks 22.23, 22.21 cc. This means that there is a slight amount of non-sugar reducing substance in the returned fluid, approximately equivalent to 0.05 mg. of glucose in 0.05 cc. or about 0.1 per cent glucose. If this is subtracted from the glucose equivalent of total reducing substances in the returned fluid we get 1.93 per cent true glucose. This is about the same as indicated by the optical activity of this fluid; *i.e.*, there is practically no other optically active substance present. There was next introduced 0.4 per cent NaCl solution, about isotonic with 2 per cent glucose. This gave a polarimeter reading of  $0.12^\circ$ , showing no mutarotation. This reading is practically the same as the zero reading, showing that very little optically active substance passes into 0.4 per cent NaCl solution in the gut. To see whether any copper-reducing substances entered the

gut in this case we took 0.5 cc. of returned fluid in 5 cc. of tungstic acid filtrate. Titrations were 21.90, 21.92 cc. Blank was 22.23, 22.21 cc., giving a titration of 0.31 cc. of 0.005 N thiosulfate due to copper-reducing substances added to the salt solution in the gut. In order to see whether this was glucose we had other samples to which yeast suspension was added. These gave titrations of 22.00, 22.02 cc.; yeast blank was 22.07, 22.13 cc. It is thus seen that practically none of the copper-reducing substances entering the salt solution in the gut is glucose. The amount of non-sugar reducing substance entering a salt solution is about the same as was found to enter the 2 per cent glucose solution. In this experiment, then, the excess of copper-reducing power over dextro-rotation in the returned fluid is due to an entrance into the gut contents of non-sugar reducing substances rather than of levorotatory substances.

We will not give the details of all the experiments but will state the percentage glucose equivalents of the returned solutions in the six experiments in terms of copper reduction value and of dextro-rotation, the first figure of each pair representing the former, the second the latter. Dog A 1.97, 1.80, 1.94, 1.86; Dog B 2.19, 2.22, 2.08, 2.10, 2.03, 1.91, 2.31, 2.20. By the steps outlined in the above protocol it was found that in both experiments with Dog A the greater copper-reducing than dextrorotatory value of the returned solutions was due to the entrance of levorotatory substances into the gut contents, for there were no non-sugar reducing substances present. With Dog B, in the two experiments where the copper reduction and dextrorotation were practically the same (2.19, 2.22, 2.08, 2.10), there were small amounts of copper-reducing substances present, approximately equivalent to 0.2 per cent glucose. Since, in spite of the presence of these non-sugar reducing substances, the ratio of reducing value to optical value of the fluids is practically the same as for pure glucose solutions, it is apparent that non-sugar dextrorotatory substances in amount equivalent to the non-sugar reducing substances have also entered the gut contents. Of the two experiments with Dog B in which the reducing value of the returned fluids was greater than their dextrorotatory value (2.03, 1.91, 2.31, 2.20) one, cited in the protocol, is explained by the addition of non-sugar reducing substances, the other by the addition of a slight



amount of levorotatory material, since the yeast filtrates in this case showed that there was no non-sugar reducing substance present. We feel sure that the differences in titration figures from which these conclusions are drawn are well within the error of the methods. Even though one may not be willing to grant that strictly quantitative glucose figures can be obtained with differences of 0.2 to 0.4 cc. between blank and unknown, still with duplicates checking usually within 0.05 cc. one can state confidently whether a given discrepancy between reducing value and optical value is due to non-sugar reducing substances or to non-sugar optically active substances.

#### SUMMARY.

In six experiments on two Thiry-Vella fistula dogs no evidence of a mutarotation of an approximately 2 per cent solution of glucose in distilled water was seen in the 30 to 40 minutes following a 10 minute stay in an intestinal loop. The readings were begun within 5 to 7 minutes after removal from the gut and were made with a sodium lamp; the solutions were cleared by a brief period of centrifugation, no chemical treatment or filtration being employed. Hewitt and Pryde's findings, from which they inferred the formation of a  $\gamma$ -glucose, are not confirmed.

In four of six experiments the copper-reducing value of the returned fluid was greater than its dextrorotatory value. In three of these this was due to an entrance of non-reducing levorotatory substances into the gut contents, in one to the entrance of non-sugar reducing substances. In the two cases where the ratio of reducing value to optical value was practically the same as for pure glucose solutions, there were added to the gut contents non-sugar reducing substances which had practically the same optical activity as has glucose.

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## UTILIZATION OF THE CALCIUM OF SPINACH.

By LAURA McLAUGHLIN.\*

*(From the Food and Nutrition Laboratory of the Bureau of Home Economics,  
United States Department of Agriculture, Washington.)*

(Received for publication, May 20, 1927.)

Spinach, rich in minerals and vitamins, is one of the most widely used of leafy vegetables. It ranks high among the vegetables in calcium, but its calcium is less readily soluble than that of most other vegetables (1). However, calcium that is insoluble in cooking water may be rendered soluble in the stomach and upper intestine and thus be utilized. It seems probable that if this calcium is utilized that of other vegetables under the same conditions would be. Again, spinach contains an unusually high percentage of oxalic acid (2) which unlike most other organic acids of fruits and vegetables seems not to be oxidized in the body (3). Eaten in large quantities, free or combined, oxalates might possibly interfere with the assimilation of food, for cases of oxalic acid poisoning from food have been reported (4). For these reasons, it was planned to compare the calcium balance on a diet in which spinach furnished a very high proportion of the calcium with that of the same diet in which milk furnished an equal proportion of the calcium.

Seven healthy women of the scientific staff of the Bureau of Home Economics acted as subjects. For 2 days previous to the experimental period and again after it until the marker in the feces appeared, the subjects ate varied diets estimated to have approximately the calcium content of the experimental period. A weighed simple mixed diet thought to contain all the body requirements and with the foods apportioned as nearly as possible like that recommended by Hunt in "Good Proportions in the Diet" (5) was eaten for 6 days. Milk furnished 79 per cent of the total calcium

\* The author was assisted by Gordon Kline, Helen Woodward, and Fanny Walker Yeatman.

intake. During the next 6 days spinach and some cream estimated to contain an equal amount of calcium and to have the same proximate composition as the milk, were substituted for it. The calcium content of this diet proved to be slightly higher, spinach furnishing 73 per cent of it.

*Diet.*—The diet consisted of round of beef, potatoes, bread made without milk or fat, Grimes Golden apples, butter, and sugar, and either milk or else spinach with cream. Five of the seven subjects drank a cup of coffee each morning; the other two drank it the last 6 days only. Except for that in the bread, no salt was used in cooking but 12 gm. of chemically pure sodium chloride were weighed out for each subject for each 3 day period to be used as desired. Distilled water was used for cooking and for drinking.

Menus were varied within each period and the food as served was appetizing. Milk and spinach were apportioned according to the weights of the subjects, but instead of thus adjusting the energy content of the diets it was thought that the varying requirements of the individuals would be better met by allowing some choice in the quantities eaten of such low calcium foods as were easily weighed if portions remained uneaten. Though the average energy contents for the two periods was the same, in the case of one individual the difference was 7 per cent. She was less active during the spinach period. Each subject was weighed at the beginning of the experiment and the morning of each 3rd day. As there was no change in weight the energy requirements were probably just met.

Milk, cream, and spinach were secured fresh each day. The spinach was cooked without added water and a composite sample of each day's supply was used for analysis. The bread was made four times a week by a uniform formula in the Milling Investigations Laboratory of the Bureau of Agricultural Economics. Enough of each of the other foods was secured at one time for the whole experiment. The beef was kept in cold storage; enough for a 3 day period was ground at a time and thoroughly mixed and sampled for analysis. Potatoes were cooked in the skin, peeled, and riced for samples. Apples were cored, or cored and pared, before weighing. Meat, potato, and apple dishes were cooked in individual glass casseroles from which the food was eaten.

*Analysis.*

*Foods.*—Sugar was considered 100 per cent pure. Nitrogen, ether extract, ash, and calcium were determined in all foods except apples and potatoes in which the ether extract was not determined because it is so low that any error introduced by computing from data from the literature was considered negligible. Oxalic acid was determined in spinach and in potatoes, and crude fiber in spinach. All analyses were made in duplicate or triplicate.

*Urine.*—24 hour collections were measured and acidity and creatinine determinations made on these samples. Aliquots were taken for composite 3 day samples and preserved under toluene in a refrigerator. After acidifying to pH 3, and filtering, they were analyzed for calcium.

*Feces.*—Feces were marked off in 3 day periods with carmine or charcoal. As soon as collected and weighed they were put on a steam bath for 24 hours in the enameled pails in which they were collected, then ground, transferred to large weighing bottles, dried to constant weight in an electric oven at 100°C., and kept in desiccators until analyzed for nitrogen, ether extract, total ash, and calcium.

*Methods.*—Ash determinations of the dried foods and feces were made by heating in platinum or vitreosil dishes in an electric muffle furnace at dull red heat. Calcium was determined in the ash of foods and of feces and in urine by McCrudden's method (6); brom-cresol green was used as the indicator for the acidity (pH 4.8) for precipitation (7), and the oxalate was titrated with potassium permanganate.

The casein of butter was determined by difference by igniting the residue from ether extractions. All other nitrogen determinations and all fat determinations were made by the Bureau of Chemistry, nitrogen by the Kjeldahl method in the Analytical Reagents Laboratory, the fat of milk by the Babcock method in the Food Control Laboratory, and other fat determinations as ether extract in the Cattle Foods Laboratory. Oxalic acid was determined by precipitation as the calcium salt.

Folin's method (8) was used for creatinine determinations in urine. The concentrations of hydrogen ion in urine were determined colorimetrically by a modification of Brown's method (9).

## RESULTS AND DISCUSSION.

Table I gives the average composition of the foods. The milk was the mixed product from six cows and showed great variation from day to day in its proximate composition as well as in the calcium content. The deviations from the mean were quite as great in spinach.

The quantity of spinach to be used had been estimated on the basis that milk and cooked spinach contain about the same percentage of calcium. The percentage of calcium in the spinach of the last 3 days was much higher than that of the previous 3 days. However, the average percentage of calcium in milk for 6 days

TABLE I.  
*Average Percentage Composition of Foods.*

Food.	Moisture.	Protein (N $\times$ 6.25).	Fat.	Ash.	Calcium.
Apple, with skin.....	83.16	0.26	(0.3)*	0.265	0.0051
“ without skin.....	84.28	0.21	(0.3)*	0.239	0.0054
Bread.....	34.20	8.29	0.16	1.361	0.0135
Butter.....	13.33	1.08	83.08	0.250	0.039
Cream.....	53.48	2.08	40.86	0.456	0.0764
Meat.....	69.06	20.55	9.23	0.978	0.0114
Milk.....	85.76	3.80	4.9	0.731	0.1338
Potato, cooked.....	77.32	2.27	(0.1)*	0.711	0.0042
Spinach, “.....	89.01	4.07	0.26	1.752	0.1322

\* From the literature.

was 0.1338, for cooked spinach 0.1322. For this reason and because these foods furnished most of the calcium, it seems better to compare the data of two 6 day periods.

There was no difference shown by the two diets in the length of time required for the appearance of the feces markers. The percentages of moisture in the feces (averages 80 and 82) showed no appreciable difference in tendency toward diarrhea or constipation.

It seems that any marked difference in accessibility of the food masses to the digestive juices would be indicated by differences in the coefficients of digestibility of the proteins and the fats. Milk protein and spinach protein each constituted about 17 per cent of the total protein of the diets. The average coefficient for the

protein of the milk period was 91.9 per cent, for that of the spinach period 88.6; the difference between the two seems small enough to be accounted for by the differences in the digestibilities of the animal and vegetable proteins. Also, the fats of the two diets were digested to the same extent (97.2 for the milk period and 97.7 for the spinach period).

TABLE II.  
*Calcium Intake and Output per Day.*

Subject.	Average intake.	Intake per kilo.	Output in urine.	Output in feces.	Total output.	Total retention.	Retention per kilo.
Milk period.							
	gm.	mg.	gm.	gm.	gm.	gm.	mg.
L. A. ....	0.456	8.8	0.066	0.300	0.366	0.090	1.7
C. C. ....	0.501	8.8	0.214	0.207	0.421	0.081	1.4
E. H. ....	0.447	8.8	0.139	0.217	0.356	0.090	1.8
A. H. ....	0.551	8.8	0.199	0.294	0.492	0.058	0.9
L. M. ....	0.449	9.4	0.160	0.235	0.395	0.054	1.1
R. S. ....	0.505	8.9	0.129	0.207	0.336	0.169	3.0
R. V. ....	0.542	8.5	0.180	0.225	0.405	0.137	2.1
Average.....	0.493	8.9	0.155	0.241	0.396	0.097	1.7
Spinach period.							
L. A. ....	0.487	9.4	0.033	0.431	0.463	0.024	0.5
C. C. ....	0.519	9.1	0.068	0.412	0.480	0.038	0.7
E. H. ....	0.452	8.9	0.087	0.357	0.443	0.009	0.0
A. H. ....	0.561	8.9	0.063	0.411	0.474	0.087	1.4
L. M. ....	0.467	9.7	0.071	0.378	0.449	0.018	0.4
R. S. ....	0.516	9.1	0.079	0.393	0.471	0.045	0.8
R. V. ....	0.558	8.7	0.079	0.404	0.483	0.075	1.2
Average.....	0.509	9.1	0.069	0.398	0.458	0.042	0.7

*Oxalic Acid.*—Boiled potatoes contained 0.070 per cent oxalic acid, and cooked spinach 0.712 per cent. In the six samples of spinach analyzed oxalic acid varied even more than did calcium; the quantities of the two were not proportional. The quantity of spinach eaten by each subject contained on the average about 2 gm. of oxalates computed as oxalic acid, a quantity, it might seem, which when repeated for 6 days would show a deleterious effect, if

there were such a tendency. No digestive disturbances were noticeable.

*Calcium Balances.*—The intake, output, and retention of calcium for each subject are given in Table II. The calcium balances for two 3 day periods when milk supplied the greater part of the calcium in quantity above the minimum requirement show retention of calcium in seven adult women. When milk calcium was replaced by that of spinach, retention of calcium continued through two more 3 day periods in six subjects in spite of the natural tendency toward equilibrium.

Marked difference is shown in the proportion of calcium eliminated through the kidneys, there being 2 to 3 times as high a percentage during the milk period. This may be attributed to the higher acidity of the urine: the average for the milk period was pH 6.1, for the spinach period pH 6.8. Milk itself has an alkaline ash so that the great decrease in acidity of the urine during the spinach period emphasizes strongly the content of base-forming elements in spinach, one effect of which is to divert the elimination of much of the calcium to the intestines (10). How much of the calcium excreted by way of the intestine was previously absorbed is not known. A continued storage shows that it was utilized.

McClugage and Mendel (11) in a balance experiment on two dogs using dried spinach found that its calcium was very poorly utilized compared with that of milk. This they thought might possibly be due to the bulk of indigestible residue in the diet because the mass of the feces in the vegetable periods was 90 to 100 per cent greater. In our experiment with human subjects and freshly cooked spinach the food was well digested. The spinach contained 0.80 per cent crude fiber. The weight of the dried feces of an individual for the spinach period was on the average about 1.4 times that of the dried feces for the milk period.

Sherman and Hawley (12) found that vegetables represented by carrots, celery, and canned spinach were a much poorer source of calcium for the growing child than is milk. Per kilo of body weight, a child's need of calcium is many times that of the adult.

Blatherwick and Long (13), using mixed vegetables furnishing about the same quantity of calcium per kilo of body weight as did our spinach, found that the calcium was well utilized. Rose (14)

found that when carrots provided 55 per cent of the calcium in the diet it was utilized as well as was the calcium of milk.

In the normal adult a tendency toward calcium equilibrium is expected. Clark (15) found that five men who had been on a prison diet stored calcium during an experimental period that varied in length from 19 to 28 weeks during which they were fed a diet containing from 11.0 to 15.6 mg. of calcium per kilo of body weight. In four of the five subjects the calcium retention was greater during the weeks when cooked fresh vegetables were included in the diet than during the weeks when there was milk but no fresh vegetables. The prolonged storage of calcium was taken as an indication that the general prison diet did not contain all the necessary foods in adequate amounts. The largest retention of calcium seemed to be by individuals whose previous food habits might account for a calcium deficiency.

In our short experimental period calcium retention probably indicates its need as well as its utilization. But a lowered retention during a spinach period following a milk period may not show that the calcium of the spinach was less utilizable. The differences in the individual balances do not seem to justify a quantitative comparison of the foods.

#### SUMMARY.

Spinach, fed for 6 days as the only food high in calcium in a diet consisting of some of the most commonly used foods produced distinctly positive calcium balances in six out of seven healthy women and calcium equilibrium in the seventh. Spinach furnished 70 per cent of the calcium, and the intake was greater than the calculated requirement for maintenance. It was thought that the repeated use of an unusually large quantity of spinach would emphasize any tendency toward hindrance to calcium assimilation through presence of fiber or oxalates, but no such tendency was demonstrated. Storage of calcium in adult women is assumed to demonstrate a utilization of the calcium of spinach.

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## THE DISTRIBUTION OF PROTEIN IN THE BLOOD IN EXPERIMENTAL ANEMIA.

By MEYER BODANSKY, STANLEY W. MORSE, VEON C. KIECH,  
AND ROBERT B. BRAMKAMP.

(From the Department of Chemistry, Stanford University, California.)

It has been brought out by Howe (1, 2) that the protein of the plasma may be separated into a number of fractions by precipitation in solutions of sodium sulfate of varying concentration. Fibrinogen is said to separate out in 0.75 molar sodium sulfate; euglobulin, Pseudoglobulin I, and Pseudoglobulin II are precipitated at concentrations of 1.00 M, 1.25 M, and 1.50 M sodium sulfate, respectively. At a concentration of 1.50 M, the globulins are presumably separated from the albumins, the latter remaining in solution. Following Howe's procedure, the albumins may be separated into five fractions. These are designated by the numerals V, VI, VII, VIII, and IX, and are precipitated respectively at the following concentrations of sodium sulfate: 1.75, 2.00, 2.25, 2.50, 2.75 M.

One object of the present work was to determine whether there is any uniformity in the distribution of the protein fractions of the plasma (or serum) of the dog as determined by Howe's method. It was appreciated that the protein fractions obtained by salting-out procedures do not necessarily represent fixed chemical entities.

### *Distribution of Protein Fractions in Plasma and Serum of Normal Dogs.*

The analyses were performed on the blood of eleven healthy dogs. The data are outlined in Table I and show that in every case the total albumin exceeded the total globulin. Of the globulin fractions, Pseudoglobulin I was usually present in greatest amount (eight analyses out of eleven). The euglobulin was relatively low in all cases, as compared with the remaining fractions.

There was little uniformity as regards the relative amounts of the two pseudoglobulin fractions.

Of the albumin fractions, Fraction VIII was usually present in greatest amount. This is of interest in view of the fact that in most animals either Fraction VI or VII is predominant. In the horse, chicken, and guinea pig, Fraction VI is greater than Fraction VII, whereas the reverse is found in man and cow, sheep and rabbit (Howe). In Dog 19, Fraction VII was somewhat greater than Fraction VIII, and in Dogs 5 and 6, albumin Fraction VI was predominant. Howe has observed similar variations in the cow with respect to Fraction VII.

In analyzing the plasma, fibrinogen was determined both by precipitation in 0.75 M sodium sulfate (Howe's method (2)) and by the method of Cullen and Van Slyke (3). The results obtained by the former procedure were invariably higher than by the method of Cullen and Van Slyke. Moreover, serum treated in 0.75 M sodium sulfate invariably yielded a small amount of precipitate which could be removed by filtration and estimated in the usual way by the Kjeldahl method. This was true, not only in the experiments reported in this paper, but in a larger series of determinations performed in another connection. As given in Table I, the values for the 0.75 fraction in the plasma analyses include fibrinogen; in the serum analyses, the 0.75 fraction represents only the small precipitate obtained by treating serum in 0.75 M sodium sulfate.

*Distribution of Protein Fractions in Serum in Acetylphenylhydrazine Anemia.*

A number of experiments was performed with the object of determining whether any change in the distribution of the protein fractions of the serum may be demonstrated in experimental anemia. Analyses were made of the serum, according to Howe's method, before and after the production of anemia, care being taken to use only blood that showed no hemolysis. Even when the degree of hemolysis is slight, the values obtained for total serum protein are high, most of the hemoglobin being removed with the albumin Fraction VI. Where hemolysis has taken place, the filtrates obtained from all precipitations up to 2.00 M sodium sulfate contain hemoglobin and are reddish in color. The remaining

TABLE I.  
*Distribution of Protein Fractions in Plasma and Serum of Dog.*

Results are expressed as gm. of protein N in 100 cc.

Dog No.	Material analyzed.	Total protein N.	Fibrinogen (Cullen-Van Slyke).	0.75 M fraction.	Euglobulin N (1.00 M).	Pseudo-globulin I N (1.25 M).	Pseudo-globulin II N (1.50 M).	Total globulin N.†	Albumin N.					Total albumin N.	Albumin/Globulin.
									V	VI	VII	VIII	IX		
2	Serum.	1.059	0.028	0.009	0.035	0.309	0.099	0.452	0.043	0.133	0.132	0.244	0.055	0.607	1.34
3	"	1.008	0.066	0.047	0.055	0.138	0.079	0.319	0.116	0.107	0.077	0.347	0.042	0.689	2.16
4	"	0.931	0.029	0.001	0.030	0.132	0.137	0.300	0.102	0.130	0.095	0.270	0.034	0.631	2.10
5	"	1.057	0.032	0.037	0.072	0.202	0.164	0.475	0.138	0.167	0.140	0.095	0.042	0.582	1.23
6	"	0.979	0.027	0.059	0.057	0.104	0.159	0.379	0.103	0.177	0.172	0.081	0.067	0.600	1.56
7	"	0.949	0.029	0.082	0.015	0.199	0.132	0.428	0.130	0.111	0.093	0.145	0.042	0.521	1.22
8	"	1.062	0.030	0.042	0.031	0.201	0.180	0.454	0.129	0.135	0.099	0.195	0.050	0.608	1.34
9	"	1.007	0.041	0.017	0.040	0.237	0.107	0.391	0.119	0.123	0.174	0.187	0.013	0.616	1.57
17	Plasma.	0.902	0.092	0.123	0.0590	0.0976	0.1721	0.3597	0.0211	0.1264	0.0584	0.1838	0.0606	0.4503	1.25
18	"	0.962	0.047	0.060	0.0950	0.2250	0.0250	0.358	0.0700	0.2450		0.2160	0.0260	0.557	1.55
19	"	1.005	0.052	0.082	0.0900	0.2150	0.0530	0.388	0.1490	0.0260	0.1900	0.1650	0.0350	0.565	1.46

\* The fibrinogen in experiments on Dogs 2 to 9 was determined by analyzing specimens of plasma taken at the time the serum was prepared for the remaining analyses.

† The values for total globulin in the plasma analyses include euglobulin, the pseudoglobulins, and the difference between the 0.75 fraction and the fibrinogen fraction as determined by the method of Cullen and Van Slyke. In the case of the serum analyses, the values given include the euglobulin, pseudoglobulins, and the small fraction precipitated in 0.75 M sodium sulfate.

TABLE II.  
Data Showing Changes in Distribution of Proteins of Serum during Anemia.

Dog No.	Date.	Red corpuscles in millions per c.mm.	Fibrinogen N in 100 cc. plasma.	Total protein N in 100 cc. serum.	Protein fractions in gm. of N per 100 cc. serum.								Total globulin N per 100 cc. serum.	Albumin/Globulin.	Remarks.		
					0.75 M	1.00 M	1.25 M	1.50 M	1.75 M	2.00 M	2.25 M	2.50 M				2.75 M	
1	1925			gm.									gm.	gm.			
	Nov. 2	6.50	0.053	0.784	0.013	0.086	0.101	0.057	0.162	0.146	0.199	0.020	0.257	0.527	2.05	Normal.	
2	" 9	1.85	0.170	0.971	0.148	0.106	0.127	0.015	0.113	0.145	0.205	0.112	0.396	0.575	1.45	Anemia.	
	Nov. 16	7.48	0.039	1.091	0.045	0.004	0.051	0.241	0.082	0.214	0.321	0.134	0.341	0.751	2.20	Normal.	
9	" 25	3.56	0.066	1.393	0.062	0.189	0.130	0.141	0.115	0.412	0.211	0.133	0.522	0.871	1.48	Anemia (hemolysis).	
	1926																
10	Feb. 15	11.70	0.032	1.008	0.013	0.048	0.281	0.120	0.113	0.044	0.322	0.067	0.462	0.546	1.18	Normal.	
	" 23	3.50	0.154	1.019	0.041	0.236	0.207	0.106	0.004	0.165	0.231	0.029	0.590	0.429	0.73	Anemia.	
11	Mar. 8	3.36	0.099	0.880	0.046	0.126	0.208	0.028	0.094	0.249	0.104	0.025	0.408	0.472	1.15	"	
	Apr. 21	5.28	0.040	0.838	0.070	0.097	0.204	0.031	0.177	0.119	0.122	0.018	0.402	0.436	1.09	Partial recovery.	
10	Mar. 15			0.984	0.012	0.110	0.178	0.135	0.138	0.086	0.282	0.043	0.435	0.549	1.26	Normal.	
	" 24	2.00	0.123	0.970	0.059	0.135	0.210	0.098	0.071	0.105	0.062	0.228	0.002	0.502	0.468	0.93	Anemia.
11	Apr. 5	5.22	0.038	0.785	0.035	0.034	0.193	0.111	0.015	0.089	0.075	0.221	0.012	0.373	0.412	1.11	Partial recovery.
	" 19	6.25	0.050	0.823	0.040	0.018	0.142	0.120	0.120	0.122	0.257	0.024	0.320	0.523	1.63		
11	Mar. 22	7.80	0.031	0.976	0.058	0.048	0.176	0.136	0.087	0.174	0.049	0.226	0.022	0.418	0.558	1.33	Normal.
	" 30	3.71	0.055	0.919	0.025	0.097	0.304	0.052	0.108	0.148	0.169	0.016	0.426	0.493	1.15	Anemia.	

filtrates in the series are water-clear. It seems that the hemoglobin is carried down with the globulin and albumin Fractions V, VI, and VII and that the precipitation limit for dog hemoglobin is about 2.25 M sodium sulfate.

The data recorded in Table II are the results of several typical experiments. It is to be noted that the albumin-globulin ratio was diminished in every case during anemia. The fibrinogen of the plasma and the 0.75 and 1.00 M (euglobulin) fractions of the serum were invariably increased during anemia. The pseudoglobulin fractions showed no consistent changes.

Even mild irritation of the liver is said to increase the formation of fibrinogen. This may account for the change in fibrinogen in our experiments, although it has been shown by one of us (4) that acetylphenylhydrazine differs from many of the hydrazine derivatives in that it is not destructive of liver tissue unless the intoxication is made chronic by continued administration of the drug (5). However, the liver is stimulated to increased activity in experimental anemia as a result of the excessive destruction of red corpuscles, and this factor may have been responsible for the increased formation of fibrinogen.

The significance of the euglobulin change is somewhat more obscure. Handovsky (6) states that there is an inverse relationship between the quantity of cholesterol which can be extracted from serum with ether and its euglobulin content. It is conceivable that the low cholesterol values obtained both in experimental and clinical forms of anemia may be due to the removal of a portion of the cholesterol by combination with protein. Gardner and Gainsborough (7) have presented evidence to show that the changes in the albumin-globulin ratio which so frequently occur in many diseases, as well as under normal physiological conditions, may be related to the variations in the sterol content of the plasma. In the light of these observations, it is possible that the apparent increase in euglobulin during experimental anemia may be due to the formation of a protein-cholesterol complex having the precipitation limits attributed to euglobulin. It may therefore be desirable to determine the fate of the cholesterol liberated from red corpuscles during their disintegration.

*Protein Fractions of the Corpuscle.*

Besides hemoglobin, the red cells of all mammals are said to contain a protein—cell-globulin  $\beta$ . This protein coagulates at 75°C. and is precipitated by partial saturation with sodium chloride, by carbon dioxide, or by dialysis. It was shown by Halliburton (8) that cell-globulin  $\beta$  is not a globulin but a nucleoprotein. Ponder (9) states that about 5 per cent of the red cell is composed of this protein. However, much lower values are given by Abderhalden (10) in two analyses of dog corpuscles. The percentage of protein in the corpuscle, other than hemoglobin, is given as 0.9918 in one analysis and 0.532 in a second analysis.

Since the precipitation limit for hemoglobin is about 2.25 M sodium sulfate, it was supposed, at first, that it might be possible to separate the cell proteins into fractions, assuming that the hemoglobin were to be removed within a limited range of concentrations of sodium sulfate. The experimental procedure employed in testing this supposition was as follows: The corpuscles were separated from the serum by centrifuging, washed repeatedly with Brinkman's solution (NaCl 0.7 per cent,  $\text{NaHCO}_3$  0.2 per cent, KCl 0.1 per cent,  $\text{CaCl}_2$  0.02 per cent, and enough  $\text{CO}_2$  to make the hydrogen ion concentration equal to  $0.45 \times 10^{-7}$ ; in this solution the cells are stable), and separated after each washing by centrifuging the suspensions. The thoroughly washed corpuscles were finally suspended in Brinkman's solution and their volume determined by means of the Daland hematocrit. The specific gravity of the suspension, as well as of the Brinkman solution, was also determined. From these data, the weight of the corpuscles in a given volume of suspension was calculated. A definite volume of suspension was then diluted with an equal volume of water. The mixture was tightly stoppered and set away in an incubator at 37°C. for several hours. This method of hemolysis (*i.e.*, hemolysis in dilute salt solution at moderate temperature) was selected as the most suitable for the purpose of this study.

The hemolyzed mixture was filtered to remove traces of cell debris. 5 cc. portions of the filtrate were added to each of a series of sodium sulfate solutions (60 cc.) of such dilution that the precipitations occurred at the following concentrations: 0.75, 1.00, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75 M. These mixtures were then set aside in an incubator (at 37°C.) for several hours, filtered, and the

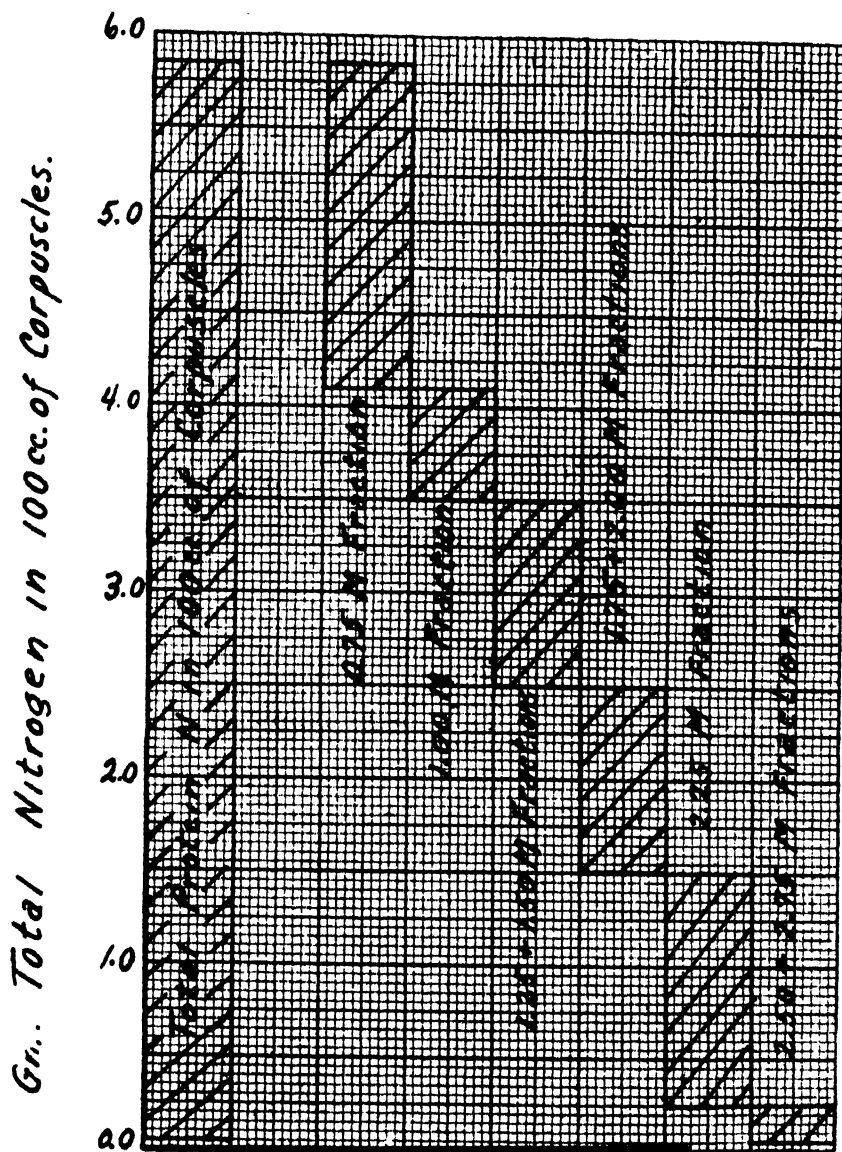


FIG. 1. Protein fractions of hemolyzed corpuscles.



filtrates analyzed for nitrogen. The various protein fractions were thus determined essentially according to Howe's method for plasma proteins. Estimations of total protein nitrogen and of non-protein nitrogen in the hemolyzed mixtures were also made.

The corpuscles of five specimens of normal dog blood were analyzed in this way, each determination being made in duplicate. There was close agreement in the five experiments, the average results of which are represented in Fig. 1. The total protein nitrogen per 100 gm. of corpuscles averaged 5.8 gm., which is equivalent to about 36.25 gm. of protein. On the basis of numerous other experiments, it may be estimated that of this quantity at least 30 or 32 gm. were hemoglobin. Actually more than 50 per cent of the total protein precipitated as globulin, and nearly 40 per cent of the total was salted out as the 0.75 fraction. Relatively little protein remained in the filtrate from the 2.25 M precipitation.

It appears, therefore, that the hemoglobin liberated from the corpuscles was altered in some way, this resulting in the precipitation of several fractions in relatively dilute salt solutions. This raises again the question of the significance of the earlier observations in which the fibrinogen and euglobulin fractions were found to increase in anemia. Hemolysis occurs after the injection of acetylphenylhydrazine, and although the anemic blood was not collected for analysis until after hemolysis had disappeared (except in Dog 2), the possibility nevertheless remains that a part of the extra protein salted out as fibrinogen and euglobulin may have had its origin in the hemoglobin of the corpuscles. It has been stated by Reymann (11) that in the process of immunization there is an increase of fibrinogen and globulin and that both of these proteins may have their origin in the globin of the red cell.

Chick (12) has demonstrated that pseudoglobulin, by combining with lecithin, is converted into an insoluble product displaying the properties of euglobulin. The addition of soap, saponin, bile, and a variety of other substances to serum results in the precipitation of "insoluble globulin," according to Jarisch (13). The work of Gardner and Gainsborough (7) shows that the presence of sterols modifies the solubility of proteins. All of these observations suggest a method for studying the apparent conversion of hemoglobin into globulin-like proteins, as well as of the more general

problem of the shift in the albumin-globulin ratio which occurs in so many diverse conditions.

#### SUMMARY.

The distribution of the protein fractions in the plasma and serum of the dog has been studied according to Howe's method. Of the globulin fractions, Pseudoglobulin I was usually predominant; of the albumins, Fraction VIII was usually present in greatest amount.

A small amount of protein is precipitated when serum from defibrinated blood is added to 0.75 M sodium sulfate.

The albumin-globulin ratio of the serum proteins was found to diminish during anemia. Invariably, the fibrinogen content of the plasma and the euglobulin fraction of the serum increased in amount, and the 0.75 M fraction of the serum was increased in nearly every case. A tendency to return to normal values was observed in several experiments in which the animals were allowed to recover.

More than half of the total protein, including hemoglobin, liberated from normal corpuscles by hemolysis in dilute salt solution may be salted out within the precipitation limits of the globulins. The significance of this observation is discussed in relation to the shift in the albumin-globulin ratio of the serum.

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## THE FATE OF SUGAR IN THE ANIMAL BODY.

### VII. THE CARBOHYDRATE METABOLISM OF ADRENALECTOMIZED RATS AND MICE.\*

BY CARL F. CORI AND GERTY T. CORI.

(*From the State Institute for the Study of Malignant Disease, Buffalo.*)

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There are several reports in the literature to the effect that insulin injections lead to an increased discharge of epinephrine. It was, therefore, of importance to study the influence of insulin on the carbohydrate metabolism of animals without, or at least with a minimum of epinephrine secretion. Rats are especially suitable for such work, since a large percentage survives double adrenalectomy for a long time. The survival of the rat, in contrast to the rapid death of other species, is generally ascribed to the presence of accessory cortical tissue undergoing hypertrophy after the removal of the main gland. On the other hand, no appreciable amount of epinephrine-producing tissue has been found in adrenalectomized rats.<sup>1</sup> Apart from rats, adrenalectomized mice have also been used for the present investigation, since it was found that they have an even better chance of survival than rats.

Cannon, McIver, and Bliss (1), using the denervated heart as indicator, found an increased discharge of epinephrine when the

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<sup>1</sup> Vincent, S., (*Internal secretion and the ductless glands*, New York and London, 2nd edition, 1922) in discussing the possible rôle of accessory medullary tissue for the survival of the rat after double adrenalectomy, states on p. 148: "It does not appear to be the case that the rat is more richly endowed with extracapsular chromophil cells than are other common animals. The present writer [Vincent] has been so far totally unable to demonstrate any such tissue by the method of Stilling and Kohn and is further informed by Dr. Kohn that there is, at any rate, no essential difference between the rat and other animals as regards its chromophil tissues."

blood sugar was reduced to a critical level by insulin. They also criticized previous negative results obtained with the vena cava pocket method by Stewart and Rogoff (2). Poll (3) and Kahn (4) demonstrated histologically a sharp drop in the epinephrine content of the adrenal medulla following insulin injections. Abe (5) found in rabbits a dilatation of the completely denervated iris after insulin. He estimated that 0.0005 to 0.001 mg. of epinephrine per kilo per minute was discharged in excess.

The majority of the workers seem to agree that a surplus of epinephrine is released during insulin hypoglycemia. In view of this, there was the possibility that some of the metabolic changes that have been ascribed to insulin were actually produced by epinephrine or by a combined action of the two hormones. This might have been the case especially in the changes in liver glycogen observed after insulin, since it is known that epinephrine causes liver glycogen to be split into glucose. It has been shown previously (6) that large doses of insulin inhibit glycogen formation in the liver. By killing groups of rats with and without insulin 1, 2, 3, 4, and 5 hours after glucose or fructose feeding, curves for the rate of glycogen formation in the liver could be constructed. The inhibiting influence of insulin injections was very marked in all cases. The blood sugar of the rats receiving glucose plus insulin ranged from 60 to 73 mg. per cent. For the 4 hour glucose absorption period a complete balance of the absorbed sugar has been made. It was found that in experiments on normal and insulinized rats with equal absorption, where 90 per cent of the absorbed glucose could be accounted for by oxidation plus glycogen formation in the liver and in the rest of the body, the amount of glucose (83 mg.) that failed to be deposited in the liver of the insulinized rats corresponded to the amount of glucose (97 mg.) that they oxidized in excess. The chief effect of insulin in these experiments, where the amount of sugar available was the same in both normal and insulinized animals, was a shift in the disposal of sugar from the liver into the muscles. The question was whether or not epinephrine played a rôle in bringing about this shift. Some evidence was presented that the increased sugar oxidation in the insulinized rats occurred chiefly in the muscles. The interpretation was that on account of the larger amount of glucose disposed of in the muscles correspondingly less glucose was left to

be stored as glycogen in the liver. However, in case an increased discharge of epinephrine were to cause less liver glycogen to be deposited by splitting the new formed glycogen into glucose, leaving a surplus of sugar to be disposed of elsewhere in the body, the interpretation of the mechanism of insulin action would be entirely different. For this reason the experiments on normal rats referred to above were repeated on adrenalectomized rats.

Insulin injections have also been shown to lead to a decrease in the liver glycogen of fasting animals. The same alternatives as in the case of the sugar-fed animals presented themselves. Either the glycogenolysis was caused indirectly by an increased disposal of sugar in the muscles or it was the direct effect of epinephrine secretion. It was, therefore, of interest to test the influence of insulin on the liver glycogen of fasting adrenalectomized animals.

#### *Adrenalectomy in Rats and Mice.*

The operations were performed under ether anesthesia by the lumbar route. Deep narcosis was avoided in all cases, since it took only 5 minutes to remove both glands. No attempt has been made to determine the ultimate percentage of survival, this being beyond the scope of the present investigation. According to Jaffe (7) 35 per cent of the operated rats die within 30 days, the major number of deaths occurring between the 5th and the 12th day. 46 per cent show a chronic suprarenal insufficiency and die within 6 to 7 months. Only 19 per cent of the adrenalectomized rats appear to be normal and seem to survive indefinitely. In our series of 60 rats only 2 died within 1 week of the operation. This is merely mentioned to show that the technique of operation was satisfactory. Beyond 1 week no definite figures can be offered.

Male rats between 120 to 150 gm. of body weight were operated upon. It was found that about 26 per cent of the animals, 7 to 14 days after the operation, had either maintained their original body weight or even surpassed it. These were the animals that were found suitable for the experiments. Rats that had shown a loss in body weight after the operation either died during the 24 hour fast preceding the experiments or died some hours after the glucose feeding.

No satisfactory data could be found in the literature on the survival of adrenalectomized mice. Out of thirty-six operated

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mice kept under observation for 22 days only six died: one each after 5, 8, 13, and 21 days, and two after 9 days. The rest seemed quite normal and continued to gain in weight.<sup>2</sup>

## *Blood Sugar and Liver Glycogen of Fasting Adrenalectomized Rats.*

Six adrenalectomized rats were used as controls for the determination of the preformed glycogen (Table I). They were fasted for 24 hours before the glycogen in the liver and in the rest of the body was analyzed, Pflueger's method being used. A surprising result was that liver glycogen was either entirely absent or that at

TABLE I.  
*Glycogen Content of 24 Hour Fasting Adrenalectomized Rats.*

Body weight.	Liver in per cent of body weight.	Glycogen in liver.	Glycogen in other tissues.	Total glycogen per 100 gm. of body weight.	Blood sugar.
<i>gm.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>
140.7	3.41	0	0.211	0.150	53
147.0	3.56	Trace.	0.234	0.159	72
153.7	3.79	0	0.198	0.129	71
122.0	3.02	0	0.166	0.136	67
151.5	2.81	0	0.231	0.152	77
152.7	3.00	Trace.	0.182	0.119	79
Average...	3.26			0.141	70
Average for 24 hr. fasting normal rats (18).....				0.150	99

best only traces could be demonstrated. At the same time the blood sugar of these animals ranged considerably below normal, namely from 0.053 to 0.079 per cent. In normal rats an average of 0.2 per cent liver glycogen has been found after a 24 hour fast with blood sugars ranging from 0.096 to 0.102 per cent. In no case was the liver of these fasting normal rats free of glycogen. A low sugar concentration in the blood of adrenalectomized cats and

<sup>2</sup> The question whether accessory cortical or medullary tissue can be found in adrenalectomized mice is being studied by Mr. Hanan from the Department of Anatomy of the University of Buffalo. The mice used for the experiments reported in this paper were turned over to him for examination and he informed us that the removal of the adrenals was complete in all cases.

dogs has been described quite regularly. Since these two species generally refuse to eat for some time before death, it seems probable that many of the blood sugar determinations were made on animals in a fasting condition.

There is good reason to link the low blood sugar of the fasting adrenalectomized rats with the absence of liver glycogen. The muscles and the other tissues of these animals were well supplied with glycogen, as is shown in Table I, but the muscle glycogen does not participate in blood sugar regulation. Bollman, Mann, and Magath (8) showed that the fall in blood sugar following hepatectomy does not lead to a marked diminution of muscle glycogen. Best, Hoet, and Marks (9) found on the eviscerated spinal preparation of the cat that a severe insulin hypoglycemia lasting for several hours does not cause a significant depletion of the glycogen stores of the muscles. The authors (10) always observed less sugar in venous than in arterial blood of the muscle, even if an extreme hypoglycemia was produced by insulin. One has, therefore, to regard the liver glycogen as the only or at least as the most important source of blood sugar.

It seems certain that the disappearance of liver glycogen in fasting adrenalectomized rats is not the result of a disturbance in the synthesis of sugar into glycogen. As will be shown later, glycogen is formed quite rapidly when glucose is fed, in spite of the fact that the liver was initially free from glycogen. Nor is there any lack of liver glycogen in non-fasting adrenalectomized rats (Schwarz (11), Kahn and Starkenstein (12), Stewart and Rogoff (13), Kuriyama (14)). There is at present no indication whether the absence of liver glycogen in fasting adrenalectomized rats is a symptom of cortical insufficiency or whether it is due to a lack of epinephrine secretion. It may be of interest in this connection that small doses of epinephrine given to animals rendered free of liver glycogen, give rise to the new formation of liver glycogen (Pollack (15), Kuriyama (16)). While large doses of epinephrine produce glycogenolysis, small doses have apparently the opposite effect. Kuriyama, however, believes that the influence of epinephrine on glycogen storage in the liver is not a specific one, since other toxic substances may behave in a somewhat comparable manner.



*Recovery Experiments with Glucose on Adrenalectomized Rats.*

The nitrogen excretion, and the amount of glucose absorbed, oxidized, and stored as glycogen in the liver and in the rest of the

TABLE II.

*Recovery Experiments with Glucose on Adrenalectomized Rats.*

All values are per 100 gm. of body weight per 4 hours.

Fore period.		Glucose absorption period.										
O <sub>2</sub>	R.Q.	Glucose absorbed.	Glucose oxidized.	Glycogen formed.			Glucose recovered.		O <sub>2</sub>	R.Q.	Urine N.	Blood sugar.
				In liver.	In rest of body.	Total.						
gm.		gm.	gm.	gm.	gm.	gm.	gm.	per cent	gm.		mg.	mg.
0.818	0.726	0.600	0.332	0.083	0.125	0.208	0.540	90.1	0.746	0.858	12.03	167
0.877	0.712	0.649	0.337	0.144	0.150	0.294	0.631	97.3	0.785	0.854	12.04	Lost.
0.745	0.727	0.925	0.384	0.182	0.158	0.340	0.724	78.3	0.878	0.855	13.76	147
0.837	0.694	0.710	0.398	0.070	0.132	0.202	0.601	84.6	0.819	0.870	11.28	168
0.808	0.725	0.839	0.413	0.139	0.166	0.305	0.718	85.6	0.872	0.865	11.73	165

TABLE III.

*Recovery Experiments with Glucose Plus Insulin on Adrenalectomized Rats.*

All values are per 100 gm. of body weight per 4 hours.

Fore period.		Glucose absorption period.										
O <sub>2</sub>	R.Q.	Glucose absorbed.	Glucose oxidized.	Glycogen formed.			Glucose recovered.		O <sub>2</sub>	R.Q.	Urine N.	Blood sugar.
				In liver.	In rest of body.	Total.						
gm.		gm.	gm.	gm.	gm.	gm.	gm.	per cent	gm.		mg.	mg.
0.835	0.720	0.869	0.388	0.078	0.185	0.263	0.651	74.9	0.851	0.861	13.00	118
0.878	0.699	0.676	0.402	0.015	0.173	0.188	0.590	87.4	0.784	0.880	13.39	55
0.890	0.719	0.722	0.448	0.017	0.168	0.185	0.633	87.6	0.690	0.924	12.88	66
0.780	0.701	0.939	0.514	0.034	0.235	0.269	0.783	83.4	0.851	0.905	10.42	93
0.979	0.726	0.934	0.603	0.040	0.167	0.207	0.810	86.7	0.944	0.917	12.26	97

body were determined on the same animal in the manner described in previous papers (17, 18). The rats were fasted for 22 hours previously. After a metabolism fore period of 2 hours, glucose was

fed by stomach tube. In one group of experiments 8 units of insulin per 100 gm. of body weight were injected simultaneously with the sugar feeding. After the absorption had proceeded for

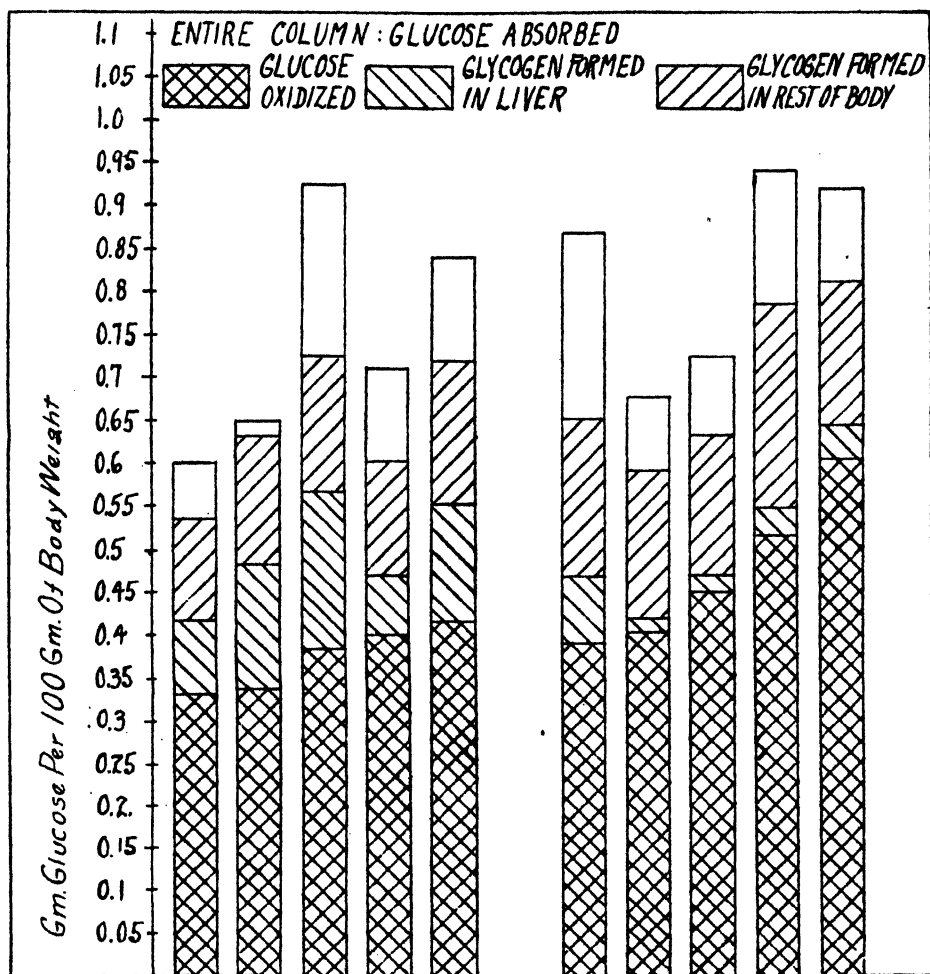


FIG. 1. Graphic illustration of the experiments in Tables II and III. Bars 1 to 5 are the experiments without and Bars 6 to 10 the experiments with insulin.

4 hours, the second metabolism period was interrupted and the animal sacrificed.

It was found that 24 hour fasting adrenalectomized rats absorbed less glucose than 24 hour fasting normal rats. In work to be published later the normal 24 hour fasting rats absorbed an

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average of 274 mg. of glucose per 100 gm. per hour against an average of 196 mg. for the operated animals here reported. Data

TABLE IV.  
*Average of Experiments in Tables II and III.*

All values are per 100 gm. of body weight per 4 hours.

	Glucose alone (5 rats).	Glucose plus insulin (5 rats).
Fore period:		
O <sub>2</sub> .....	0.817 gm.	0.872 gm.
CO <sub>2</sub> .....	0.810 "	0.861 "
R.Q.....	0.718	0.713
Total calories.....	2.80	3.09
Glucose absorption period:		
Glucose absorbed .....	0.745 gm.	0.828 gm.
"    oxidized .....	0.373 "	0.471 "
Glycogen formed.....	0.270 "	0.222 "
Glucose recovered.....	0.643 "	0.693 "
	(86.3 per cent).	(83.7 per cent).
"    lost.....	0.102 gm.	0.135 gm.
Glycogen formed.....	0.72	0.47
Glucose oxidized.....		
Blood sugar.....	0.162 gm.	0.086 gm.
O <sub>2</sub> .....	0.820 "	0.824 "
CO <sub>2</sub> .....	0.976 "	1.022 "
R.Q.....	0.860	0.897
Urine N.....	12.17 mg.	12.39 mg.
Non-protein O <sub>2</sub> .....	0.717 gm.	0.719 gm.
"    CO <sub>2</sub> .....	0.862 "	0.906 "
"    R.Q.....	0.870	0.911
Protein oxidized.....	0.075 gm.	0.076 gm.
Fat .....	0.111 "	0.076 "
Calories from protein*.....	0.30	0.31
"    "    fat†.....	1.05	0.71
"    "    glucose‡.....	1.40	1.76
Total calories.....	2.75	2.78

\* Heat from protein (urine N × 24.98 calories).

† Heat value for animal fat 9.4 calories.

‡ Heat value for glucose 3.74 calories.

presented in a previous paper (18) showed that 48 hour fasting normal rats absorb decidedly less fructose than 24 hour fasting normal rats. The same is true for glucose, since the 48 hour fasting rats

absorb only 180 mg. per 100 gm. per hour. One may infer from this that the rate of intestinal absorption is influenced to a marked extent by the general physical condition of the animal. It is, therefore, of significance that the adrenalectomized rats, though they were fasted for only 24 hours, are close to the 48 hour fasting normal rats as regards the rate of glucose absorption.

The individual experiments are recorded in Tables II and III and are illustrated graphically in Fig. 1. An average of the experiments has been calculated in Table IV. It will be noted that the average amount of glucose absorbed per 100 gm. of body weight per 4 hours was 745 mg. for the animals without and 828 mg. for the animals with insulin. This corresponds to a difference in absorption of 83 mg. or of 10 per cent. Though the absorption does not check as closely as in previous experiments on normal animals, the difference is not a serious one, since the amount of sugar oxidized is within these limits independent of the amount of sugar absorbed. It has been observed repeatedly that the rats absorbing most do not necessarily oxidize the largest amount of sugar and *vice versa*. The increase in sugar oxidation in the insulinized animals is, therefore, regarded as significant. The average amount of glucose oxidized was 373 mg. for the animals without and 471 mg. for the animals with insulin. The recovery of the absorbed glucose was 86.3 per cent for the former and 83.7 per cent for the latter animals.

The starting point for the present investigation was the inhibiting effect of insulin injections on the glycogen formation in the liver of normal rats. The following results have been obtained on animals with intact adrenals. During 4 hours of glucose absorption the animals without insulin deposited 3.68 per cent liver glycogen against 1.14 per cent for the animals receiving insulin. Calculated per 100 gm. of rat this corresponds to a difference of 83 mg. of liver glycogen. For an equal amount of sugar absorbed the insulinized rats oxidized 97 mg. more glucose than the animals without insulin, which more than accounts for the difference in liver glycogen. The average blood sugar for the insulinized animals was 77 mg. per cent. The question was whether or not the same results would be obtained on animals with both adrenals removed in view of the fact that insulin hypoglycemia has been shown to lead to an increased discharge of

epinephrine. The argument was that if the failure of the insulinized normal rats to deposit liver glycogen was actually caused by epinephrine, adrenalectomized rats receiving insulin should not show a lessened deposition of liver glycogen. If, on the other hand, epinephrine were not involved, the effect of insulin on the liver glycogen should be the same in both normal and adrenalecto-

TABLE V.

*Amount of Glycogen Formed in Liver and in Rest of Body in 24 Hour Fasting Adrenalectomized Rats during 4 Hours of Glucose Absorption.*

	Liver in per cent of body weight.	Gly- cogen in liver.	Gly- cogen in other tissues.	Total gly- cogen.	Liver in per cent of total gly- cogen.	Other tissues in per cent of total glycogen.
		gm.	gm.	gm.		
Glucose alone.....	3.89	0.124	0.146	0.270	45.9	54.1
“ plus insulin.....	3.78	0.037	0.185	0.222	16.6	83.4
Difference.....		-0.087	+0.039	-0.048		

mized animals. This argument presupposes that by removing both adrenals not enough medullary tissue remains in the body to give rise to an appreciable epinephrine production and that the loss of cortical tissue does not lead to a change in carbohydrate metabolism.<sup>3</sup> Table V contains the average glycogen values for the adrenalectomized rats calculated from Tables II and III. The animals without insulin deposited 124 mg. of liver glycogen

<sup>3</sup> Histological studies on rats by Vincent and others, referred to in the introduction, tend to show that the rat has the same if not less accessory medullary tissue than the common laboratory animals. The workers who use the denervated heart or iris as indicator of epinephrine discharge, perform control experiments after removal or denervation of the adrenal glands. They regard it as proof that a certain experimental procedure has caused an increased discharge of epinephrine, if this experimental procedure leads to an acceleration of the heart beat or to a dilatation of the iris in the presence of the adrenals, but fails to have this effect in the absence of the adrenals. Their assumption is, of course, that by removing the adrenals a negligible amount of epinephrine-producing tissue is left in the body. If, on the other hand, the effect on heart rate and size of the iris persists after removal of the adrenals, they do not feel justified in concluding that epinephrine is involved. Exactly the same line of argument underlies the interpretation of our experiments on rats.

against 37 mg. for the animals receiving insulin injections. This corresponds to a difference of 87 mg., or, expressed per 100 gm. of liver, to 3.19 and 0.98 per cent glycogen respectively. The result that less liver glycogen is deposited following insulin, in spite of the fact that the adrenals had been removed, makes it unnecessary to assume that epinephrine is responsible for this phenomenon. Table IV shows that the difference in glucose oxidation between the animals with and without insulin is 98 mg. in favor of the former. Hence, the difference in oxidation corresponds closely to the difference in liver glycogen, which is in complete analogy to the results obtained on animals with intact adrenals.

The evidence presented tends to show that the metabolic changes observed in insulinized animals in the type of experiment here described are due to insulin alone and not to a combined action of insulin plus epinephrine. This does in no way contradict the results of those workers, who obtained an increase discharge of epinephrine during insulin hypoglycemia. In the first place, the hypoglycemia in our experiments might not have been strong enough to cause a release of epinephrine and, secondly, the amount of epinephrine that produces a definite effect on the denervated heart or iris might not be large enough to cause liver glycogen to be split into glucose.

Recent experiments, to be reported later, have some bearing on the second point. When 0.02 mg. of epinephrine was injected simultaneously with the glucose feeding, about 50 mg. of glucose were excreted in the urine. A complete balance of the absorbed glucose showed that this glycosuria was not caused by a failure of the liver to store glycogen; that is to say, the amount of liver glycogen formed was the same in the animals with and without epinephrine. If the glycosuria of 50 mg. would have been caused by a diminished glycogen formation in the liver, the difference in liver glycogen would have been of about the same magnitude as in the insulin experiments referred to above. An effective dose of epinephrine leading to a marked sugar excretion in the urine is without influence on glycogen storage in the liver, while insulin inhibits glycogen formation in the liver even in adrenalectomized animals. This is considered additional evidence for our contention that the diminished glycogen formation in the liver of insulinized animals is not caused by epinephrine.

*Influence of Insulin on Liver Glycogen of Fasting  
Adrenalectomized Mice.*

The idea has been prevalent among workers in the insulin field that the organism endeavors to counteract by some sort of regulatory mechanism the fall in blood sugar accompanying insulin injections. When Dudley and Marrian (19) had shown that insulin leads to a decrease in the liver glycogen of fasting animals, it was assumed that the liver glycogen was mobilized for the purpose of blood sugar regulation. There was but one step to the further assumption that an increased discharge of epinephrine was the means of effecting the glycogen mobilization. This reasoning has been put to the test by investigating the influence of insulin on the liver glycogen of fasting adrenalectomized mice.

The mouse has been chosen as the experimental animal, partly because this species survives double adrenalectomy in a large percentage of the cases, partly because the influence of insulin on the liver glycogen of fasting mice with intact adrenals had been investigated on a former occasion (20). In this previous work sixteen insulinized mice showed, as an average, 0.9 per cent liver glycogen and 66 mg. per cent blood sugar, while sixteen control mice that were treated in exactly the same way (except for the insulin injection) and were killed simultaneously with the injected mice showed 1.47 per cent liver glycogen and 131 mg. per cent blood sugar. The present series of experiments on adrenalectomized mice comprises fourteen injected and fourteen control animals. Following the operation the animals were fed on dog biscuits plus bread. In the previous series, on normal mice only dog biscuits were fed. The addition of bread to the diet probably accounts for the higher liver glycogen of the control mice of the present series. Otherwise the experimental procedure was the same in the two series. After a preliminary fasting period of 1 to 5 hours one-half of the mice was injected intraperitoneally with 0.01 to 0.04 units of insulin. 35 to 45 minutes later, before any outspoken hypoglycemic symptoms occurred, the injected mice were killed and the livers of two to three mice combined for the determination of the glycogen according to Pflueger's method. An equal number of control mice were killed simultaneously and worked up in the same way. The livers were removed as quickly

as possible, frozen with compressed CO<sub>2</sub>, minced, and transferred into boiling KOH. Blood sugar was determined on each mouse separately. An examination of the stomach and the intestines showed that these animals were in a postabsorptive state when

TABLE VI.

*Influence of Insulin on Liver Glycogen of Fasting Adrenalectomized Mice.*

The animals were used 20 to 30 days after operation. Control and injected mice were killed simultaneously, the livers of two to three mice being combined. I, injected; C, control.

Body weight.	Length of time of fasting.	Insulin dose (in clinical units) and time of killing.	Blood sugar.	Gly- cogen per 100 gm. liver.
gm.	hrs.		mg.	gm.
I. 20.8 21.2 23.3	1	40 min. after 0.01 units.	98 77 102	1.73
C. 21.6 22.3 22.4	1		159 145 143	2.87
I. 22.7 23.6 25.2	2	45 " " 0.02 "	56 76 97	0.69
C. 21.1 24.2 25.3	2		151 138 141	2.91
I. 25.3 26.2	2	35 " " 0.04 "	87 92	1.14
C. 25.4 26.1	2		135 146	2.29
I. 25.9 27.1	4	45 " " 0.04 "	84 93	0.75
C. 26.1 26.6	4		114 124	1.47
I. 26.3 27.0	4	45 " " 0.04 "	76 81	0.24
C. 26.6 26.9	4		136 157	2.65
I. 24.8 25.9	5	35 " " 0.04 "	64 62	0.21
C. 25.1 25.3	5		123 136	0.78

Average blood sugar of insulinized mice, 0.082 per cent.

" " " " control " 0.139 " "

" liver glycogen " insulinized " 0.79 " "

" " " " control " 2.16 " "

they were killed. The experiments were performed 20 to 30 days after adrenalectomy.

The data recorded in Table VI show in a clear cut way that insulin injections lead to a reduction in the liver glycogen of fasting adrenalectomized mice. It will be noted that the injected mice



had an average of 0.79 per cent liver glycogen against 2.16 per cent for the control mice. This is a substantial reduction, if one considers that the hypoglycemia was only of a moderate degree. The blood sugar of the insulinized mice ranged from 56 to 102 mg. per cent, with an average of 82 mg. per cent. Hypoglycemic symptoms were not observed at these blood sugar levels. Previous work (20) on mice with intact adrenals has shown that convulsions do not occur until the blood sugar has fallen to 40 to 45 mg. per cent. Roughly speaking, there was no marked difference in the sensitivity of the normal and adrenalectomized mice against insulin as regards the rapidity of fall in blood sugar and the blood sugar level at which symptoms occurred. Though these observations cover a period of only 35 to 45 minutes after the injection, one has to consider that convulsions in insulinized mice may occur as soon as 20 minutes after the injection. It is, of course, possible that a difference might have been detected if the minimal lethal dose of insulin for normal and adrenalectomized mice had been worked out.

If adrenalectomized mice are fasted for 9 hours or longer, the liver glycogen disappears and the blood sugar falls below normal as in the case of the 24 hour fasting adrenalectomized rats. In three adrenalectomized mice receiving no insulin typical convulsions supervened after 5 hours of fasting. One animal was injected with glucose and recovered promptly. The other two mice were killed and showed blood sugar values of 32 and 43 mg. per cent, respectively, and a complete absence of liver glycogen.

The main result is that the effect of insulin on the liver glycogen of fasting mice is the same, whether or not the adrenals are present. One may infer from this that the decrease in liver glycogen following insulin is not due to epinephrine acting on the liver glycogen. Excluding epinephrine, there remain several other possibilities by which the decrease in liver glycogen might be explained. These will be taken up in the "Discussion."

#### DISCUSSION.

Several workers in the insulin field have suggested in personal communications to the authors that some of the results obtained in the second and third papers of this series (6, 17) might have been due to a combined action of insulin plus epinephrine, the

latter being discharged in excess on account of the insulin hypoglycemia. What was meant was the inhibition of glycogen formation in the liver of animals receiving glucose plus insulin and the decrease in liver glycogen in fasting insulinized animals. It may not be amiss to state again the experimental conditions under which these results have been obtained.

The first point is that the rats with and without insulin have the same amount of sugar available and have, therefore, to dispose of the same amount of sugar. This is due to the fact that insulin does not influence to any marked extent the rate of absorption of sugar from the intestine. Secondly, in the rats with and without insulin the amount of glucose absorbed is almost entirely accounted for by sugar oxidation plus glycogen formation in the liver and the rest of the body (chiefly muscles). If an injection of insulin (or of any other substance) is to show an effect at all under these conditions, this can only be by influencing one of the two processes of sugar disposal at the expense of the other. This follows from the fact that the amount of sugar absorbed as well as recovered is the same in the rats with and without insulin. If, for instance, insulin were to accelerate sugar oxidation, the glycogen formation would have to be decreased to the same extent as the oxidation is increased. As has been stated in the introduction, what actually happens is that the increase in sugar oxidation following insulin corresponds very closely to the decrease in glycogen formation in the liver. This means that the glycogen deposition in the muscles is not materially changed.

In describing this effect of insulin on normal rats one is justified in speaking of a shift in the disposal of sugar from the liver into the muscles; that is to say, the muscles appropriate so much of the absorbed sugar that almost no sugar is left to be stored as glycogen in the liver. The point under discussion is how this shift is brought about. Large doses of epinephrine are known to lead to glycogenolysis in the liver. The question whether or not epinephrine is responsible for this shift has been tested in two ways. In the first place, the experiments on normal rats have been repeated on adrenalectomized rats with the result that exactly the same shift occurred when insulin was given. Secondly, 0.02 mg. of epinephrine has been injected simultaneously with the sugar feeding. Though sugar was excreted in the urine, there was no lessened deposition of glycogen in the liver.

In analyzing the evidence here presented, the conclusion has been reached that epinephrine is not involved in the metabolic changes that have been ascribed to insulin. The effects observed were those of insulin alone and not of a combined action of insulin plus epinephrine. There is, therefore, no need for the writers to alter their conception of the mechanism of insulin action, as would have been necessary if epinephrine had been found to play a metabolic rôle.

The chief action of a surplus of insulin is seen in intensification of the normal processes of sugar disposal; that is, of oxidation plus glycogen storage. Though insulin acts in the same way, whether it is injected into sugar-fed, into fasting, or into diabetic animals, the effects to be observed are different, mainly, because the relation between the amount of sugar and insulin present varies in each of these cases. Under physiological conditions, as in the normal sugar-fed rat, the relation between the amount of sugar absorbed and the amount of insulin released by the pancreas is such that liver glycogen is formed very rapidly (apart from glycogen deposition in the muscles and oxidation). For 100 parts of absorbed glucose, 38 parts are oxidized, while 36 parts are deposited as glycogen in the muscles and 16 parts in the liver. On the other hand, if the amount of sugar absorbed remains the same but the insulin content of the tissues is increased, as in the sugar-fed and insulinized rat, only 4 out of 100 parts of absorbed sugar are deposited as liver glycogen. By disturbing the physiological relation between sugar and insulin, the disposal of sugar in the muscles is increased to such an extent that most of the sugar is appropriated by them with the result that less liver glycogen is deposited and that the blood sugar falls below normal.

Attributing to insulin no other action in the fasting than in the sugar-fed animal, the effects to be observed in the former animals following an overdose of insulin can easily be deducted. The hormone injection leads to an increased disposal of sugar in the muscles. This causes the blood sugar to fall. The low blood sugar sets up a demand for sugar, which can be met for a time by the preexisting carbohydrate reserves of the body. The main carbohydrate reserves for the purpose of blood sugar regulation are those of the liver. Soon the liver glycogen begins to diminish and is sometimes completely exhausted before convulsions super-

vene. The convulsions lead to a further depletion of the muscle glycogen, so that the animal may die with all its initial glycogen reserves used up. In the present paper the question how liver glycogen is mobilized under these conditions has been investigated. When insulin was injected into fasting adrenalectomized mice, the liver glycogen diminished in the same way as in mice with intact adrenals. The conclusion was that epinephrine is not responsible for the mobilization of liver glycogen during insulin hypoglycemia. If it is not epinephrine, it might be by way of the sympathetic system that glycogenolytic impulses are sent to the liver, since stimulation of the liver nerves has been shown to cause carbohydrate mobilization. This possibility has not been investigated so far. From a metabolic view-point, a possible discharge of epinephrine in sufficient quantities to produce a definite metabolic effect, has been of more concern to the writers. The nature of the metabolic effect of small doses of epinephrine in sugar-fed rats will be discussed in a later publication. On this occasion an antagonism between insulin and epinephrine of a hitherto unsuspected nature will also be discussed.

Best, Dale, Hoet, and Marks (21) expressed the view that in fasting and insulinized rats or mice, with their rapid metabolism, the carbohydrate reserves of the liver and of other organs soon become exhausted, when to the increased sugar oxidation there is added the effect of a depressed new formation of carbohydrates. Laufberger (22) had previously suggested that the stoppage or restriction of the new carbohydrate formation from fat was the sole effect of insulin. According to Dale and collaborators this process alone is not sufficient to explain all the observed effects of insulin action. A promotion of oxidation and glycogen synthesis by insulin has to be assumed as well. Dale and collaborators, however, believe that the former process plays quantitatively a greater rôle in fasting animals of a smaller size such as rats and mice. In their experiments on the eviscerated spinal preparation of the cat a new formation of carbohydrates could no longer take place. Their deductions are based on experimental results published in the literature, where insulin has been shown to cause a depression of the total respiratory metabolism associated with a rise in the respiratory quotient, the fall in metabolism being explained by the restriction of new carbohydrate formation in the liver. Previous to Dale, Hawley

and Murlin (23) have made experiments which throw some light on this situation. They injected a non-convulsive dose of insulin into fasting rabbits and determined the metabolism for 2 to 4 consecutive hours. In the 2nd hour there was a sudden and very marked increase in carbohydrate oxidation. Then, with a rising blood sugar in the 3rd and 4th hour, the carbohydrate oxidation returned again to its low basal level. The important point is that the increase in sugar oxidation in the 2nd hour was associated with a fall in oxygen consumption. The diminished oxygen absorption was not due to a depression but to the fact that additional oxygen was made available for oxidation through a change in the incidence of metabolism from the oxygen-poor fat to the oxygen-rich carbohydrate. The observed difference in oxygen consumption between the basal and second insulin period in several instances exactly balanced the additional oxygen made available, the latter corresponding to the difference in the oxygen content of the materials oxidized in the two periods. In the 2nd hour the heat production fell, on an average, by 9 per cent, but returned again to the basal level in the following hours. The question arises what is the significance of this fall in heat production when the metabolism is suddenly shifted from fat to carbohydrates as the result of a surplus of insulin. Does it indicate, as Dale and collaborators believe, that the carbohydrate formation from fat is restricted or does it have some other meaning? The changes in the protein metabolism were so small that they can be neglected. On an average, 1.15 gm. of glucose replaced 0.54 gm. of fat in the metabolism. This is not an isodynamic replacement, since 1.15 gm. of glucose is the heat equivalent of only 0.45 gm. of fat. In order to balance the fall in heat production in the second insulin period an additional oxidation of 0.09 gm. of fat, representing 0.85 calories, would have been required. The best case that can be made out in support of the contention of Dale and collaborators would be to say that insulin prevented the oxidation of 0.09 gm. of fat.<sup>4</sup> There is, at present, not sufficient

<sup>4</sup> Since the respiratory quotient remains the same irrespective of the intermediary stages through which the fat molecule passes on its way to complete oxidation, such intermediary stages, as for instance conversion into carbohydrate, need not enter into the present discussion. There is also no indication for an incomplete oxidation of fat during insulin action.

evidence for the assumption that insulin inhibits fat oxidation. A fall in heat production in previously fasted animals is entirely consistent with a change in the incidence of the metabolism from fat to carbohydrates and is not dependent on an insulin injection. The authors observed a more or less pronounced decrease in heat production when sugar was fed to fasting rats, even though no insulin was given. Only when the sugar absorption had been going on for more than 4 hours did the heat production rise above normal, the rise corresponding to the so called specific dynamic action of carbohydrates.

A distinction has to be made between a temporary fall in heat production such as occurred in the experiments of Hawley and Murlin where the animals recovered spontaneously and a more pronounced fall that takes place when the animals lapse into insulin coma. When fasting animals are recovering spontaneously from an insulin injection a reversal of the metabolism takes place. As soon as the effect of insulin on carbohydrate oxidation subsides, the metabolism is shifted back to fat. The animal has returned to its normal fasting state and is none the worse for the insulin injection except that part of the carbohydrate reserves has been used up. But suppose that the influence of insulin on carbohydrate oxidation does not cease and that the supply of sugar from the carbohydrate reserves becomes suddenly very low. The organism behaves as if it were plentifully supplied with sugar, while actually the demand for sugar can no longer be met. The result will be a further fall in blood sugar and the onset of convulsions and coma. Under such conditions it is perhaps not surprising that the animals finally die with a low body temperature. The greatly reduced metabolism of animals in insulin coma may be attributed to the depressing effect of a low blood sugar on the function of the nervous centers. Indeed, Holmes and Holmes (24) have adduced some evidence that the carbohydrate metabolism of the brain is dependent to a large extent on the glucose supplied to it by the blood. When the blood sugar falls below a critical level the first effect of a low supply of glucose is an irritation of the nervous centers resulting in convulsions. The second stage is a paralysis of the nervous centers resulting in coma and in a fall in heat production. It should be noted that the low rate of metabolism of comatose animals is nothing specific, since coma from other causes than insulin hypoglycemia also leads to a reduction of

the total respiratory metabolism. Why, then, assume that a restriction in the new formation of carbohydrates is responsible for the low metabolism, as has been done by Dale and collaborators? On the basis of the experimental evidence available so far the authors believe that there is no need to ascribe to insulin any other metabolic action besides its well recognized effect on carbohydrate oxidation and storage. The question of new formation of carbohydrates will be discussed in more detail in a later publication. There is one clear case in which insulin, though indirectly, inhibits new formation of carbohydrates. This is the depancreatized or completely phlorhizinized animal, in which the protein metabolism is excessively high with wasteful formation of sugar from protein. The oxidation of sugar that is setting in after the insulin injection exerts its well known sparing action on protein metabolism.

#### SUMMARY AND CONCLUSIONS.

1. When rats surviving double adrenalectomy were subjected to a 24 hour fast, the liver glycogen disappeared and the blood sugar fell considerably below normal. In contrast to the liver glycogen, the glycogen content of the muscles of these animals remained the same as that of normal control rats fasted for the same length of time. The low blood sugar is linked with the lack of liver glycogen, since the muscle glycogen does not participate in blood sugar regulation. The absence of liver glycogen in fasting adrenalectomized rats is not the result of a disturbance in the synthesis of sugar into glycogen, since liver glycogen is formed at a normal rate when glucose is fed.

2. 24 hour fasting adrenalectomized rats absorbed glucose at a much slower rate than 24 hour fasting normal rats.

3. During 4 hours of glucose absorption the adrenalectomized rats *without insulin* oxidized 373 mg. of glucose and formed 270 mg. of glycogen (124 mg. in the liver and 146 mg. in the rest of the body). The adrenalectomized rats *receiving insulin* injections oxidized 471 mg. of glucose and deposited 37 mg. of glycogen in the liver and 185 mg. of glycogen in the rest of the body. The difference in liver glycogen of 87 mg. corresponds closely to the difference in glucose oxidation of 98 mg., while the glycogen deposition in the rest of the body (chiefly muscles) is not materially changed. The

same results have been obtained previously on rats with intact adrenals, where a discharge of epinephrine in sufficient amounts to produce a metabolic effect could not be excluded. Since the adrenals were absent in the present experiments, the conclusion has been drawn that the lessened deposition of liver glycogen and the increased sugar oxidation, also to be observed in insulinized rats with intact adrenals, are due to insulin alone and not to a combined action of insulin plus epinephrine.

4. Previous experiments have shown that insulin injections lead to a decrease in the liver glycogen of fasting insulinized mice. An identical result has been obtained on mice from which both adrenals had been removed 20 to 30 days previously. The adrenalectomized mice without insulin showed 2.16 per cent liver glycogen after a fasting period of 1 to 5 hours. The adrenalectomized and insulinized mice that were killed simultaneously showed only 0.79 per cent liver glycogen. Since the effect of insulin on the liver glycogen of fasting animals is the same, whether or not the adrenals are present, there is no need to assume that epinephrine is responsible for the decrease in liver glycogen following insulin injections.

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## THE PRODUCTION OF ACETYLMETHYL CARBINOL BY CLOSTRIDIUM ACETOBUTYLICUM.

BY P. W. WILSON,\* W. H. PETERSON, AND E. B. FRED.

(From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.)

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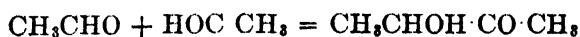
In a previous paper (1) it was reported that qualitative tests showed the production of acetylmethyl carbinol ( $\text{CH}_3\text{CHOH}\cdot\text{CO}\cdot\text{CH}_3$ ) from more than twenty different carbohydrates by the acetone-butyl alcohol-producing organism *Clostridium acetobutylicum* (Weizmann).

A quantitative study of the production of this compound was made at various stages during the fermentation in order to determine some of the important factors which accelerate or retard its development. A knowledge of the production of acetylmethyl carbinol as related to the other fermentation products, acetone and butyl alcohol, should give a better insight into the general mechanism of the fermentation and perhaps give some suggestion as to the occurrence of intermediate compounds in the decomposition of the carbohydrate.

Harden and Norris (2) found that *Bacillus lactis aerogenes* could form acetylmethyl carbinol from such widely differing substances as glucose, citric acid, and dihydroxyacetone. They showed that a synthesis must be involved in its formation and found that, when acetaldehyde was added to the culture, the reduction product of acetylmethyl carbinol, 2, 3-butylene glycol ( $\text{CH}_3\text{CHOH}\cdot\text{CHOH}\cdot\text{CH}_3$ ) was formed. Harden (3) also proved that the eosin-like color formed on adding alkali to certain cultures (Voges-Proskauer test) was due to the oxidation of acetylmethyl carbinol to diacetyl ( $\text{CH}_3\text{CO}\cdot\text{CO}\cdot\text{CH}_3$ ) and a reaction between this compound and the peptone of the medium.

\* Research Fellow of the Commercial Solvents Corporation, Terre Haute, Indiana.

Neuberg and his associates (4-8) have made an extensive study of the formation of acetylmethyl carbinol by yeast and have shown that it originates by an acyloin synthesis from 2 molecules of acetaldehyde.



By adding various aldehydes to a mass of yeast cells and sugar he was able to form several acyloin compounds and attributed the condensations to the presence of an enzyme which he named carboligase. He regarded the formation of acetylmethyl carbinol as evidence for the existence of acetaldehyde as an intermediate compound in the decomposition of the glucose.

Elion (9) has recently shown that acetylmethyl carbinol could be formed from ethyl alcohol by yeast cells if the culture was well aerated. It is probable that the alcohol was oxidized to acetaldehyde which then underwent an acyloin synthesis (10).

Inorganic salts also may affect the production of acetylmethyl carbinol. Kluyver, Donker, and Visser't Hooft (11) found that yeast produced more acetylmethyl carbinol on inorganic media than on organic nutrients.

Lemoigne (12) has developed a delicate and characteristic test for this product depending on its conversion into dimethylglyoxime with subsequent precipitation as the nickel salt. He has published a number of papers (13) on the quantity of acetylmethyl carbinol formed by various bacteria and found that it varied from 10 mg. to over 10 gm. per liter depending upon the type of organisms and the age of the culture.

More recently Donker (14), in a publication on the biochemical aspect of the butyric acid bacteria, obtained from 200 to 750 mg. of acetylmethyl carbinol per liter of culture with the butyl alcohol-forming butyrics but none or only traces with the butyrics which do not form butyl alcohol.

#### EXPERIMENTAL.

*Quantitative Method.*—The results reported herein were obtained in general from a fermentation of 8 per cent corn mash of known moisture content, inoculated with a strain of *Clostridium acetobutylicum* designated by the laboratory number of 70. Fer-

mentations were carried out at 37°C. and analyses made of the usual products, total solvents, and acetone as reported in previous publications (15). The results given are averages of duplicate fermentations, all of which checked closely.

For estimation of the acetylmethyl carbinol, Lemoigne's method, as modified by Kluyver, Donker, and Visser't Hooft (11), was employed. 200 cc. of fermented mash were distilled with 20 cc. of a 45 per cent solution of ferric chloride; 100 cc. of the distillate were collected and refluxed for 10 minutes with a mixture of 10 cc. of a 20 per cent solution of hydroxylamine hydrochloride, 20 cc. of a 20 per cent solution of sodium acetate, and 5 cc. of a 10 per cent solution of nickel chloride. In the strongly buffered solution, the diacetyl formed by the oxidation of the acetylmethyl carbinol during the distillation reacts with the hydroxylamine to form dimethylglyoxime, the latter being precipitated and weighed as the nickel salt.

Literature consulted gave no data as to the accuracy of the method or sources of error. Investigations were made, therefore, to establish the reliability of this method. It was found that addition of larger quantities of ferric chloride did not change the quantity of acetylmethyl carbinol recovered from a solution; that varying the time of refluxing or the point at which the nickel salt was added had no effect on the results. The other solvents present in the fermentation (acetone, butyl alcohol, and ethyl alcohol) did not interfere with the determination provided an excess of hydroxylamine hydrochloride was present to react with the acetone. The losses on distillation were about 5 per cent and fairly constant. It was found that by conducting the distillation so that about 40 minutes were required to give 100 cc. of distillate, maximum recovery of acetylmethyl carbinol was obtained. Duplicate determinations checked usually within 2 per cent and never differed more than 5 per cent. A sample of diacetyl (Eastman Kodak Company) that had a boiling point of 87.5–89.5°C. was analyzed by this method and a recovery of from 70 to 75 per cent was obtained. This would indicate that the method is not completely quantitative, but since it gives constant results regardless of the quantity present it is undoubtedly reliable. For absolute estimation of acetylmethyl carbinol it would be necessary to apply a correction factor to the results, but for comparative results, the

TABLE I.

*Production of Acetylmethyl Carbinol in Fermentation of Corn Mash by Clostridium acetobutylicum.*

Age of culture.	Flask 1.		Flask 2.		Flask 3.	
	Acidity.*	Acetyl-methyl carbinol.	Acidity.*	Acetyl-methyl carbinol.	Acidity.*	Acetyl-methyl carbinol.
hrs.	cc.	mg. per liter	cc.	mg. per liter	cc.	mg. per liter
15	4.5	26	4.3	34	3.6	25
21	4.5	138	4.3	70	3.8	57
27	3.5	167	3.2	126	2.8	131
39	2.8	247	3.1	145	2.4	179
44	2.7	283	3.0	163	2.2	192
50			3.2	164	2.5	192
63			3.4	192	2.3	195
72			3.3	187	2.8	189
92	3.5	308	3.2	188	2.8	180

\*Cc. 0.1 N NaOH per 10 cc. of culture.

TABLE II.

*Relation of Acetylmethyl Carbinol Production to Formation of Solvents.*

Time.	Fermentation 1.			Fermentation 2.			Fermentation 3.		
	Acidity.*	Solvents.	Acetyl-methyl carbinol.	Acidity.*	Solvents.	Acetyl-methyl carbinol.	Acidity.*	Solvents.	Acetyl-methyl carbinol.
hrs.	cc.	gm. per liter	mg. per liter	cc.	gm. per liter	mg. per liter	cc.	gm. per liter	mg. per liter
8	1.7	1.61	57	1.9	0.55	30	1.8	0.87	25
12	3.2	1.26	42	3.7	0.74	47	3.3	0.66	36
16	4.3	0.65	52	4.0	1.07	63	4.3	0.59	55
20	4.2	1.77	115	4.0	3.15	113	4.0	2.26	88
24	4.0	3.55	173	3.2	5.49	230	3.5	4.24	182
28	2.9	7.51		2.5	10.74		2.4	9.03	
32	2.6	11.17	246	2.5	14.67	274	2.5	13.13	220
36	2.8	16.10	269	2.4	18.95	276	2.6	17.84	238
50	2.7	22.60	271	2.9	22.90	236	2.6	23.28	210

\* Cc. 0.1 N NaOH per 10 cc. of culture.

method as it is, appears to be trustworthy. In this report the results are the ones actually obtained since little would be gained by multiplying them by a common correction factor.

Where phosphates were added to the fermentation mixture, it

was found necessary to remove these with  $\text{BaCl}_2$  before distilling with ferric chloride; otherwise the latter is precipitated as ferric phosphate and the acetylmethyl carbinol is not oxidized to diacetyl.

*Production during Fermentation.*—Experiments were made during which the formation of acetylmethyl carbinol was investigated by determining the amount present at different stages of the fermentation. The results given in Tables I and II and Fig. 1

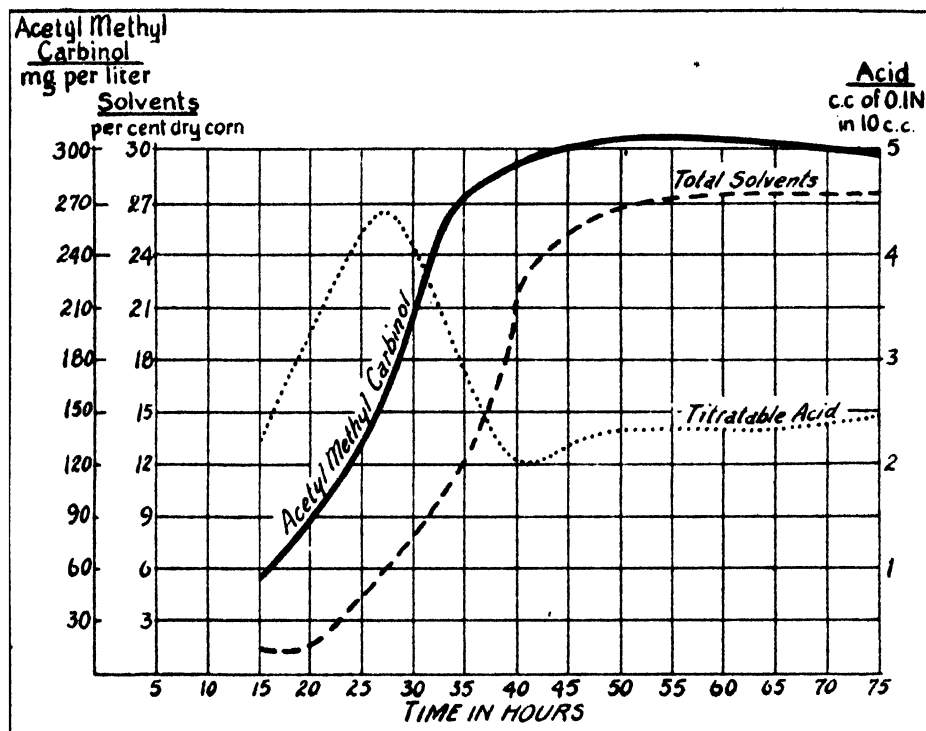


FIG. 1. The simultaneous production of acetylmethyl carbinol, solvents, and acids in the acetone-butyl alcohol fermentation.

show that this compound is an end-product of the fermentation as the quantity present increases throughout the fermentation. Its production curve follows closely that of the other solvents (acetone and butyl alcohol, etc.). However the data indicate that its maximum rate of production starts a few hours before the break in the acidity occurs; the formation of solvents reaches a maximum rate only after this break in acidity. In the majority of these experiments the quantity of acetylmethyl carbinol present reached

a maximum about 6 to 10 hours before the solvent production was ended. These data suggest that the production of acetylmethyl carbinol is more closely connected with the formation of the acids in the fermentation than with solvent formation.

*Variability of Cultures.*—The activity of the butyl alcohol organism has been reported (16, 17) to vary with the number of the subculture. In order to determine the variability in the production of acetylmethyl carbinol by different subcultures, triplicate fermentations were carried out using the second, third, and fifth subcultures of Strain 70. The results given in Table III show that the production of acetylmethyl carbinol by *Clostridium aceto-*

TABLE III.

*Variability of Culture in Respect to Production of Acetylmethyl Carbinol.*

Culture No.	No. of transfers from spore tube.	Acetylmethyl carbinol.	Solvents. (Basis of dry corn.)	Acetone.	
				Percentage of dry corn.	Percentage of total solvents.
70	2	<i>mg. per liter</i>	<i>per cent</i>		
		312	24.2	7.75	32.6
		346	25.2	8.30	32.9
70	3	400	26.1	8.30	31.8
		457	26.0	7.21	27.7
		397	25.6	8.30	31.6
70	5	366	25.7	8.15	31.0
		344	25.2	8.20	32.5
		347	25.3	8.00	31.6
		378	27.7	8.94	32.2

*butylicum* is fairly constant. However, there is some difference between triplicates even though the fermentation takes place under apparently identical conditions. This variation is about 20 per cent at the maximum, but is usually less than 10 per cent. Because of this variability, results that differed by less than 20 per cent were not regarded as significant. In two other fermentations of the fifth subculture, the results of which are not given in Table III, the range for triplicate flasks was from 363 to 415 mg. and from 360 to 388 mg. Attention is called to the fact that the triplicate fermentations which gave the maximum variation in acetylmethyl carbinol production also showed differences in the

production of total solvents or in the acetone-total solvent ratio. This observation was borne out in subsequent experiments in which these products were experimentally changed.

*Stability of Acetylmethyl Carbinol during Fermentation.*—In a recent paper Paine (18) reported that certain strains of the colon aerogenes group destroyed acetylmethyl carbinol while other strains did not. Unfortunately his results are based entirely on

TABLE IV.

*Effect of Added Acetylmethyl Carbinol on Production of Acetylmethyl Carbinol.*

Age of culture.	Acetylmethyl carbinol.				
	Flask 1.	Flask 2.		Flask 3.	
	Control.	Total.	Increase.	Total.	Increase.
<i>hrs.</i>	<i>mg. per liter</i>	<i>mg. per liter</i>	<i>mg. per liter</i>	<i>mg. per liter</i>	<i>mg. per liter</i>
Series I.					
0	0	279		279	
23	120	337	58	368	89
45	211	354	75	422	143
70	211	397	118	416	137
Series II.					
0	0	101		52	
24	195	224	123	216	164
48	274	379	278	357	305
96	296	357	256	357	305
Series III.					
0	0	332		170	
24	76	369	37	227	57
48	164	410	78	331	161
96	190	416	84	350	180

the old Voges-Proskauer qualitative test, and are therefore less conclusive than if they were based on quantitative data. Our results with the acetone-butyl alcohol organism indicate that acetylmethyl carbinol is not destroyed during the fermentation but is an end-product of fermentation. Cultures were set up in which varying quantities of acetylmethyl carbinol were added before sterilization and inoculation. Determinations were made periodically during the fermentation to observe any increase or decrease in the



amount present. The data are given in Table IV and show that acetylmethyl carbinol does not disappear during the fermentation. All flasks to which additions were made contained more of

TABLE V.  
*Effect of Adding Protein on Production of Acetylmethyl Carbinol.*

Treatment.	Final acidity.*	Acetyl-methyl carbinol.	Solvents. (Basis of dry corn.)	Acetone.	
				Percentage of dry corn.	Percentage of total solvents.
	cc.	mg. per liter	per cent		
Series I.					
Control, corn mash 8 per cent. ....	2.4	344	24.9	7.60	30.7
Plus corn gluten:†					
10.7 per cent. ....	3.2	207	24.5	7.87	32.2
21.4 " " ....	3.7	190	25.7	8.23	32.1
42.8 " " ....	3.8	47	23.5	7.80	33.3
64.2 " " ....	8.4	0	5.2		
Series II.					
Control, corn mash 8 per cent. ....	2.6	296	24.8	7.91	31.9
Plus wheat gluten:†					
10.7 per cent. ....	3.4	300	24.6	7.53	30.7
21.4 " " ....	3.9	247	25.1	8.07	32.2
42.8 " " ....	4.3	244	24.0	7.78	32.8
64.2 " " ....	5.3	182	24.4	8.18	33.5
Series III.					
Control, corn mash 8 per cent. ....	2.4	394	24.7	7.70	31.3
Plus peptone:†					
3.75 per cent. ....	3.0	481	23.2	7.32	31.5
7.5 " " ....	3.3	373	24.7	7.89	32.0
15.0 " " ....	3.5	437	24.7	7.87	31.8
30.0 " " ....	3.9	187	26.0	8.52	32.4

\* Cc. 0.1 N NaOH per 10 cc. of culture.

† Percentage based on weight of corn in culture.

the compound than the controls. When the quantity added did not exceed one-half of that found in a normal fermentation the increases were approximately equal to that formed in the con-

trol. Larger additions, however, reduced the amount formed by fermentation.

### *Modifying Factors.*

From the data given in Table III it was noted that changes in the ratio of acetone to total solvents in general resulted in corresponding changes in the amount of acetylmethyl carbinol. Changes in the acetone-solvents ratio can be brought about by the addition of buffers such as proteins and phosphates (15). The effect of such additions on the production of acetylmethyl carbinol was therefore investigated.

*Effect of Adding Protein.*—Protein was added to the fermentation mixture in three forms—corn gluten, wheat gluten, and peptone. As shown by Table V small quantities of protein do not appear to affect the fermentation. No change occurred in the acetone-solvent ratio and but little rise took place in the acidity. Only a small increase in acetylmethyl carbinol was effected by the addition of small quantities of peptone. However, larger quantities of added protein caused a rise in the acetone-solvent ratio and a decrease in the quantity of acetylmethyl carbinol. It is of interest that this decrease in acetylmethyl carbinol occurred only in those fermentations that showed a rise in acetone production.

*Effect of Adding Starch.*—The carbohydrate-protein ratio was varied in a fermentation mixture from 7.0 to 17.4 by adding starch to corn-meal. There was little effect on acetylmethyl carbinol production. Neither was the acetone-total solvent ratio changed. This experiment bears out the observation previously made that only when external factors are changed sufficiently to modify the fermentation is the production of acetylmethyl carbinol affected. In this experiment despite the added starch there seemed to be sufficient protein in the corn-meal to supply the needs of the organisms and little change in the fermentation was noted. Similarly in the experiment on adding protein small amounts do not seem to affect the fermentation, but act more or less as an inert material. Effects are observed only when large quantities of the protein are added.

*Effect of Phosphates.*—Previous work has shown that the presence of phosphates (15) in fermentations by the butyl alcohol organism lowered the acetone-total solvent ratio. The accom-

panying change in acetylmethyl carbinol production was studied by adding varying amounts of potassium acid phosphate as well as a neutral mixture of mono- and disodium phosphate to ferment-

TABLE VI.  
*Effect of Adding Phosphates on Production of Acetylmethyl Carbinol.*

Treatment.	Final acidity.*	Acetyl-methyl carbinol.	Solvents. (Basis of dry corn.)	Acetone.	
				Percentage of dry corn.	Percentage of total solvents.
	cc.	mg. per liter	per cent		
Series I.					
Control.....	2.5	389	24.8	7.57	30.7
Plus acid phosphate:†					
3.5 per cent.....	4.5	467	23.8	7.10	29.8
7.0 " ".....	7.0	533	21.1	6.20	29.4
10.5 " ".....	9.0	543	19.6	5.92	30.0
14.0 " ".....	11.4	444	20.4	6.21	30.4
Series II.					
Control.....	2.6	367	27.4	8.64	31.6
Plus neutral phosphate mixture:†					
3.5 per cent.....	3.9	675	27.5	7.33	26.6
7.0 " ".....	5.4	658	27.2	7.06	25.8
10.5 " ".....	7.1	627	26.7	6.80	25.4
14.0 " ".....	8.7	524	26.2	6.50	24.8
Series III.					
Control.....	2.2	360	24.2	7.48	31.0
Plus neutral phosphate mixture:†					
3.5 per cent.....	3.7	354	27.3	8.90	32.5
7.0 " ".....	5.4	526	27.1	8.28	30.5
10.5 " ".....	6.4	661	27.2	7.86	28.9
14.0 " ".....	8.6	758	26.6	7.17	27.0

\* Cc. 0.1 N NaOH per 10 cc. of culture.

† Percentage based on weight of corn in culture.

tations. Results given in Table VI show that as the ratio of acetone to total solvents decreases, the production of acetylmethyl carbinol increases. This increase is very striking, in some cases being over 100 per cent. In this experiment, the acetone-solvents

ratio was changed to a greater degree than when protein was added and changes in acetylmethyl carbinol were more apparent. Coincident with the reduction in the yield of acetone there was a corresponding increase in the production of ethyl alcohol.

*Effect of Calcium Carbonate.*—A number of fermentations was carried out in the presence of calcium carbonate and the effect upon the production of acetylmethyl carbinol noted. The results given in Table VII show that the addition of small amounts of calcium carbonate (1 per cent) caused a marked decrease in the production of acetylmethyl carbinol with a slightly less decrease

TABLE VII.

*Effect of Adding CaCO<sub>3</sub> on Production of Solvents and Acetylmethyl Carbinol.*

Treatment.	Solvents, dry corn.	Acetone.		Acetylmethyl carbinol.	
		Percentage of dry corn.	Percentage of total solvents.	mg. per liter	mg. per gm. of solvents
Control.....	23.2	7.40	31.9	344	21.2
Plus CaCO <sub>3</sub> :*					
1 per cent.....	14.5	4.85	33.5	171	17.2
2 " ".....	6.25	1.46	23.3	141	32.3
3 " ".....	5.96	0.63	10.5	145	35.1
4 " ".....	3.05	0.44	14.5	148	69.5
1.25 " "†.....	12.40	4.27	34.4	268	31.2

\* Percentage based on volume of culture added before inoculation.

† Percentage based on volume of culture, 0.25 per cent added 0, 16, 20, 24, 28 hours after inoculation.

in the production of solvents. Large amounts of calcium carbonate caused little further decrease in acetylmethyl carbinol but rapid decrease in solvent production. This is added proof in support of the assumption that the acetylmethyl carbinol is formed in connection with the acids rather than with the solvents.

#### DISCUSSION.

In all of the experiments in which the fermentation was modified, it appears that any change in the course of the fermentation has a greater effect on the yield of acetylmethyl carbinol than on any other product. For example the maximum decrease in acetone

production upon addition of phosphates is about 20 per cent while the increase in acetylmethyl carbinol ranges from 50 to 100 per cent. The addition of large amounts of protein increases acetone production 5 per cent or less while it decreases acetylmethyl carbinol production about 50 per cent.

This suggests that the wide differences in acetylmethyl carbinol noted in duplicate fermentations are due to slight variations in the fermentation, as a whole, and these variations are too slight to be noted in such products as total solvents, acetone, or acidity, but are detectable in the production of acetylmethyl carbinol.

The results obtained by adding calcium carbonate to the fermentation mixture indicate that the production of acetylmethyl carbinol is more closely associated with the formation of the acids than with the solvents themselves. By adding varying amounts of  $\text{CaCO}_3$  to fermentation mixtures it is possible to depress the yield of solvents in proportion to the amounts of  $\text{CaCO}_3$  added. However there is no such related depression in the production of acetylmethyl carbinol; after a preliminary depression, possibly due to change in reaction, the production of acetylmethyl carbinol is not affected by additions of more  $\text{CaCO}_3$ . It appears that the acetylmethyl carbinol is formed as a side reaction with the production of the acids from a common precursor. The amount of acetylmethyl carbinol formed is very small as compared with the acids and seems to be dependent on the pH at which the fermentation takes place. Experiments in which acetylmethyl carbinol was added before fermentation show that the quantity formed seems to have a definite limit, and that only about 50 per cent of this limit is produced in normal fermentations. In support of this view on the formation of acetylmethyl carbinol coincident with the acids are the results obtained on the production of acetylmethyl carbinol at different stages of the fermentation. These show that during the early part of the fermentation, coincident with the rapid rise in acidity, about 40 to 50 per cent of the total acetylmethyl carbinol is formed while only 10 to 15 per cent of the solvents are produced. Also, the production of acetylmethyl carbinol reaches a maximum and constant level at a time when only about 75 per cent of the solvents have been formed.

## SUMMARY.

1. Acetylmethyl carbinol is produced in the butyl alcohol fermentation as a regular end-product. The amounts formed average from 300 to 400 mg. per liter and appear to have a fairly definite limit.

2. Acetylmethyl carbinol is formed at about the same time as the acids, acetic and butyric. It is probable that all have a common precursor.

3. Acetylmethyl carbinol is more sensitive to modifying factors than the solvents, acetone and butyl alcohol.

4. The production of acetylmethyl carbinol can be increased by the addition of phosphates and decreased by the addition of proteins.

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# THE NITROGENOUS CONSTITUENTS OF HEN URINE.

By RUSSELL E. DAVIS.

(From the Laboratories of Agricultural Chemistry and Physiology, the Ohio State University, Columbus.)

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Analyses of urine from birds have been made by Minkowski (1), Milroy (2), Paton (3), Sharpe (4), and Steel (5); nevertheless in no case has the complete partition of urinary nitrogenous compounds been reported. We have collected such data. It was thought in the beginning that the total urinary nitrogen in bird droppings might be calculated from one constituent, *viz.* uric acid. Such a calculation would be advantageous in the determination of fecal nitrogen in bird droppings, a quantity that is necessary in the determination of digestibility coefficients for protein. Unfortunately our results, taken with those of Steel (5) who analyzed the white portion of bird droppings, indicate that considerable re-absorption of urinary constituents, NaCl, urea, and creatine, occurs in the cloaca of the hen. It appears impossible therefore to calculate the fecal nitrogen with any accuracy from the total and uric acid nitrogen values of bird droppings. The difficulty in obtaining bird urine, uncontaminated with feces, was overcome by using a special technique applicable to unanesthetized, unoperated birds.

Five young hens were used in this experiment. They were kept in cages throughout the entire time, and were fed the same standard ration that is used on the University poultry farm. This ration contains the following per 530 pounds.

	lbs.
Wheat middlings.....	100
Wheat bran.....	100
Corn.....	200
Meat scrap.....	100
Bone meal.....	20
Salt.....	5
Cod liver oil.....	5



The average weight of the chickens at the beginning of the experiment was 2 kilos. They grew well, gaining an average of 1.2 kilos per bird. They were normal to all appearances.

The first step in the work was to develop a method by which the urine could be collected without contamination with feces. Several investigators have done this by operating upon the birds, separating the urodeum and proctodeum, and forming an artificial anus. The urine and feces were then collected in rubber bags. An objection to this method is that the bowels become inactive and it is necessary to wash out the feces at each collection. The birds cannot be considered normal after such an operation has been performed and there are possibly some effects of trauma.

The procedure used here in the collection of urine was as follows: The hen was given 100 cc. of water by stomach tube and immediately fastened to a specially constructed chicken board. The feces were removed from the cloaca and the cloaca was carefully washed out. A catheter was then inserted into the urodeum near the openings of the ureters. The urine was collected in graduated test-tubes so the rate of flow could be easily determined. At times it was necessary to plug the intestine with cotton to prevent small pieces of feces from coming into the cloaca. Usually, however, this was not necessary unless the period of collection was over 2 hours.

The urine was analyzed for total nitrogen, uric acid, urea, ammonia, creatine, and creatinine. Creatine and creatinine were determined together and reported as creatinine. Total nitrogen was determined by the Kjeldahl method, urea by the Van Slyke and Cullen modification of Marshall's method (6), ammonia by Folin's aeration method (7). Folin's colorimetric methods (8) were used to determine uric acid, creatine, and creatinine.

The first collections of urine were made from hens that were anesthetized with ether. Such urine was dilute and the rate of excretion was very rapid. It was noted that as the effect of the anesthetic wore off the rate of flow decreased. Table I shows the effect of ether on the rate of flow and on the nitrogen content of the urine.

The hens remained quiet during the collection so that it was not necessary to use an anesthetic, but when none was used the urine was so concentrated that the solids precipitated and clogged the

catheter. To overcome this difficulty, water was given by mouth immediately before the collection of the urine. Water increased

TABLE I.  
*Effect of Ether Anesthesia on Urine Flow.*

Ether anesthesia.				No ether.		
Hen No.	Urine.	N per 100 cc.	Total N per hr.	Urine.	N per 100 cc.	Total N per hr.
	cc. per hr.	mg.	mg.	cc. per hr.	mg.	mg.
1	60	45	27.0	14.3	178	25.5
2	55	48	26.4	12.0	184	22.1

TABLE II.  
*Nitrogenous Constituents in 100 Cc. of Hen Urine and Their Proportions.*

Hen No.	Total N.		Uric acid N.		Urea N.		Ammonia N.		Creatine-creatinine N.		Undetermined N.		Remarks.
	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent		
1 a	101.2	65.0	64.2	11.2	11.0	17.2	16.9	7.5	7.4	0.3	0.29	Ether anesthesia.	
b	102.3	63.3	61.7	11.5	11.2	17.0	16.6	8.2	8.0	2.3	2.3		
c	105.0	73.0	69.5	11.6	11.0			8.9	8.8				
d	107.5	65.6	61.0	11.8	10.9	19.4	18.0	9.2	8.6	1.6	1.5		
2 a	69.4	44.8	64.6	7.6	10.2	11.8	17.0	5.0	7.1	0.2	0.3		
b	94.5	61.0	65.5	8.7	9.2	16.1	17.0	7.1	7.5	0.8	0.84		
3 a	158.0	95.0	60.0	16.3	10.2	25.4	16.0						
b	109.0	69.7	64.0	12.0	11.0	16.5	15.1	8.9	8.1	1.9	1.7		
c	85.1	53.1	62.4	8.2	9.6	14.6	17.2	5.7	7.9	2.5	2.9		
4 a	95.4	56.7	59.4	11.0	11.5	17.7	18.5	7.6	8.0	2.4	2.5		
b	101.6	67.0	66.0	9.6	9.4	15.1	14.9	8.2	8.1	0.5	0.49		
5 a	82.5	51.0	62.0	8.6	10.4	14.1	17.1	6.5	7.9	2.3	2.7		
b	126.0	74.9	59.5	13.8	10.9	24.6	19.5	10.2	8.1	2.5	2.0		
c	63.8	38.5	60.0	6.85	10.6	12.1	19.0	5.8	9.1	0.55	0.86		
Average.	100.0	62.9	62.9	10.4	10.4	17.3	17.3	8.0	8.0	1.4	1.4		

the flow of urine but did not affect the relative amounts of nitrogenous constituents.

A number of analyses was made to determine the effects of diuresis produced by ether and water on the relative amounts of the nitrogen compounds in the urine. No effect was found. The diuretics caused merely a dilution of the urine and had no effect on the nitrogenous constituents. Ambard and Wolf (9) report that water diuresis had no effect on the excretion of uric acid.

The volume of urine which is emptied into the cloaca is rather large. In these experiments the amount excreted when no diuretic was used varied greatly, the maximum being about 30 cc. per hour. If this rate is maintained for the entire 24 hours, the daily excretion would be about 700 cc. This value agrees with those obtained by Paton (3) and Sharpe (4) by direct measurement of urine from birds which had been operated upon. When diuretics were used, the flow was increased. Most of the water, therefore, which is excreted with the urine is reabsorbed in the cloaca.

The urine was neutral to litmus in every case tested. The specific gravity was determined on one sample and was found to be 1.004. In most cases it was impossible to determine the specific gravity or the reaction of the urine due to the fact that the urine was run into a solution of lithium carbonate to prevent the precipitation of uric acid.

Table II gives the results of analyses of urine samples which were collected as described above. In nearly every case the collection period was 2 hours. When these data are expressed in percentage of total nitrogen, it is seen that there is not a great deal of variation in the distribution of nitrogen waste products among the different samples. Of the total nitrogen, an average of 62.9 per cent is in the form of uric acid, 10.4 per cent in the form of urea, 17.3 per cent in the form of ammonia, and 8.0 per cent in the form of creatine-creatinine. Minkowski (1) found from 60 to 70 per cent of the total nitrogen present in the urine of normal geese in the form of uric acid, from 3 to 4 per cent in the form of urea, and from 9 to 18 per cent in the form of ammonia.

The amount of creatinine in the urine was too small to be determined by the method used but the amount of creatine was relatively large. This peculiarity of bird urine has been observed by Paton (3). Creatine was determined by converting it to creatinine by boiling with picric acid and determining the latter.

Chlorides were determined in a few samples. The amount of

chlorine found in the urine is much greater than the amount excreted in the combined urine and feces. Chlorides, therefore, are reabsorbed with water in the cloaca.

#### SUMMARY.

In fourteen trials with five hens, the uric acid nitrogen of hen urine varied from 59.4 to 69.5 per cent of the total urinary nitrogen, with an average of 62.9 per cent. Ammonium nitrogen accounted for 17.3 per cent, with variations between 14.9 and 19.5, urea nitrogen for 10.4 per cent, with variations between 9.2 and 11.2, and creatine plus creatinine nitrogen for 8.0 per cent of the urinary nitrogen with variations between 7.1 and 9.1 per cent. Uric acid nitrogen plus ammonia nitrogen accounted for 80.3 per cent of the urinary nitrogen with variations between 76 and 82.5 per cent. Since an unknown amount of reabsorption of urea, creatine, and creatinine probably occurs in the cloaca of the hen, it does not seem practical, at least without further information, to calculate urinary nitrogen from the amount of uric acid in hen droppings by multiplying uric acid nitrogen by some factor.

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## THE METABOLISM OF SULFUR.

### XIII. THE EFFECT OF ELEMENTARY SULFUR ON THE GROWTH OF THE YOUNG WHITE RAT.\*

BY GEORGE T. LEWIS AND HOWARD B. LEWIS.

*(From the Laboratory of Physiological Chemistry, Medical School,  
University of Michigan, Ann Arbor.)*\*

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In this laboratory we have been interested in the physiological rôle of substances containing sulfur which might possibly be able to influence the requirements of cystine for purposes of growth in the young white rat. Thus we have been able to demonstrate that cystine combined as in a peptide (diglycyl- or dialanyl-cystine) was utilized effectively for growth (1), while neither the dianhydride of dialanyl-cystine (1), nor the oxidation products of cystine, cysteinic acid and taurine (2), could replace cystine for purposes of growth under the experimental conditions used by us. In continuation of this line of work, we have studied the effect on the rate of growth of the white rat of the addition of elementary sulfur (flowers of sulfur) to two types of diets in which cystine was the first limiting factor.

The addition of elementary sulfur to a diet deficient in cystine did not permit maintenance in adult white mice (3). In this series, in which only 3 animals were used and the experiments extended over very short periods (1 to 2 weeks), sulfur was added to the extent of 1 per cent of the diet. No toxic effects were recorded.

From the theoretical standpoint, any influence of elementary sulfur on the requirements for or utilization of cystine might be the result of either or both of two processes. Sulfur might under-

\* The material presented in this paper and in Papers XI and XII of this series (1, 2) represents an abstract of a thesis presented by George T. Lewis in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

go transformation to some organic derivative and play a rôle in the actual synthesis of cystine in the body. Such a reaction would seem unlikely in view of the studies on the synthesis of various other essential amino-acids by the intact organism. More probable would be an effect of elementary sulfur on metabolism as a result of its conversion to sulfides, and the oxidation of the latter to sulfates by the body (4). This extra sulfate sulfur accumulated in the system might possibly act as a cystine sparer by depressing the rate of oxidation of the mercapto groups. Such an effect would have an analogy in the reported depression of the level of general protein catabolism by alteration of the rate of deamination if ammonium salts or urea are present in excess in the organism (5). Mass action of ingested ammonium salts or urea, products of deamination of the amino acids of the protein molecule, may prevent deamination of some amino acids and thus ammonium salts may function as protein spacers. Similarly sulfates, normal products of the oxidation of the sulfur of the cystine molecule, if present in amounts greater than normal, might depress the catabolic reactions in which cystine is concerned. On the basis of this theory, the addition of sulfates to a cystine-deficient diet might be expected to influence favorably the rate of growth. In the experiments of Daniels and Rich (6) however, the addition of inorganic sulfates to the basal diet did not alter the rate of growth of white rats maintained on a diet deficient in its content of cystine.

Our results have not only failed to demonstrate any favorable influence of sulfur when substituted for cystine at various levels in the diet of the white rat but have shown that continued oral administration of relatively small amounts of sulfur may produce slower rate of growth, definite toxic results, and in some cases, death of the experimental animals.

#### EXPERIMENTAL.

The experiments were carried out as described in our earlier publications (1, 2). Two types of basal cystine-deficient diets were used, the milk powder-starch diet of Sherman and Merrill (7) and the low protein (casein) diet of Osborne and Mendel (Table I). To these basal diets were added varying amounts of elementary sulfur (Baker's powdered flowers of sulfur), 0.08, 0.5,

0.75, and 1.0 per cent. Our earlier experiments were carried out with the higher percentages of sulfur and demonstrated, as already noted, a retardation of growth and marked toxicity.

In a series of controls, cystine (0.3 per cent) was supplied to demonstrate that addition of cystine to the unsatisfactory diets resulted in good growth. Since the amount of sulfur added to the diet in the earlier experiments far exceeded that added in the form of cystine in the control experiments, in a later series, an amount of sulfur equivalent to the sulfur content of cystine in the control cystine series (0.08 per cent sulfur) was added. It was felt that if sulfur could be utilized, this type of diet might be more favorable than those diets to which relatively large amounts

TABLE I.  
*Composition of Diets.*

Sherman-Merrill diet.		Osborne-Mendel diet.	
	Basal.		Basal.
	gm.		gm.
Corn-starch.....	81.8	Casein.....	9.0
Milk powder.....	16.6	Salt mixture.....	4.5
Sodium chloride.....	1.6	Corn-starch.....	54.5
		Sucrose.....	4.5
		Lard.....	24.5
		Cod liver oil.....	3.0

Sulfur and cystine were added to these basal diets as indicated in the text.

of sulfur (viewed from the standpoint of their equivalents in cystine) were added.

A considerable number of the rats were killed after periods of 9 to 14 weeks and the glycogen contents of the livers were determined according to the method of Pflüger. After hydrolysis of the glycogen, the glucose was estimated colorimetrically (Folin-Wu). The livers, kidneys, and in a few cases, the spleens of a number of sulfur rats, as well as those of the controls, were submitted to the Department of Pathology for examination. We are indebted to Professors A. S. Warthin and Carl V. Weller for the pathological diagnoses.



## DISCUSSION.

The series of rats fed the Sherman-Merrill diet included 10 litter units, a total of 73 animals. In order to condense the data, details concerning one litter only are presented in the tables. The most noticeable effect of the sulfur was that, during the first 2 or 3 weeks of the sulfur feeding, growth was much retarded or ceased altogether. Although this may have been due in part to diminished food consumption, this does not explain entirely the failure to grow. Usually the rats lost weight during the 1st week and then gained slowly, so that by the end of the 2nd week they had regained their initial weight. From this time, the rate of growth was nearly as rapid as that of the control rats on the cystine-deficient diet, but the total gain over a period of 7 to 9 weeks was not as great, since the rats fed sulfur never overcame the initial handicap of the first 2 weeks. The results suggest that the animals may have gradually acquired a tolerance for sulfur and that growth was possible only when such a tolerance was established.

Sulfur in as low a concentration as 0.5 per cent noticeably retarded the rate of growth. When the amount of sulfur added to the basal diet was increased to 0.75 per cent, retardation of growth was more marked. Thus in Litter 18, three control rats gained 60.5, 65.5, and 68.0 gm. respectively in 74 days as compared with gains of 45.0 and 47.0 gm. for their litter mates which received the basal diet with the addition of 0.75 per cent sulfur.

When the sulfur content of the diet was increased to 1 per cent, toxicity was more marked and of twenty-two animals, fourteen died in periods of from 3 to 21 days. None of the control animals in our entire series died during the course of the experiments. If an animal was able to overcome the toxic effect of the sulfur for the first 2 or 3 weeks, a tolerance for the sulfur was apparently developed and growth then occurred although at a slightly lower rate than in the control animals. It should be noted also that of the animals receiving small amounts of sulfur in the diet, one on a level of 0.5 per cent sulfur died in 29 days and two on the 0.75 per cent level died in 11 and 27 days respectively.

Since the basal diets were deficient in cystine, it seemed possible that the toxic action of the sulfur might be increased by the lack

of cystine and the consequent poor state of nutrition. We have accordingly supplemented the basal diet with 0.3 per cent of cystine and at the same time added 1.0 per cent of sulfur. This litter appeared to be especially susceptible to the toxic effect of the sulfur. All the rats which received sulfur died in 3 days; those which received cystine in addition survived no longer than their litter mates to whose diet cystine was not added.

Pathological examination of the liver and kidneys showed that the liver was the organ mainly affected. With a few exceptions, the livers of the rats which died showed a marked peripheral zonal necrosis. When the sulfur was not fed in sufficiently large

TABLE II.  
*Litter 23. Sherman-Merrill Diet.*

Period, 6 weeks.

Rat No.	Diet.	Initial weight.	Final weight.	Gain.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
226	Basal.	43.5	73.5	30.0
227	"	40.5	61.0	20.5
228	"	39.0	70.0	31.0
229	" and sulfur 0.08 per cent.	44.0	72.0	28.0
230	" " " " " "	41.0	59.5	18.5
231	" " " " " "	38.5	66.0	27.5
232	" " " " " "	39.0	53.0	14.0
233	" " cystine 0.3 " "	32.0	77.5	45.5
234	" " " " " "	33.5	83.5	50.0
235	" " " " " "	35.0	82.5	47.5

amounts to cause death, the livers showed some pathological changes but not the typical zonal necrosis. The kidneys did not exhibit any constant pathological condition.

The results with Litter 26, which received an amount of sulfur (0.08 per cent) equivalent to the cystine which effectively supplements the defective basal diet, are presented in Table II. No marked toxicity was observed and with the exception of one animal (Rat 232) the rats receiving the sulfur increased in weight nearly as rapidly as did the control animals. Addition of cystine to the basal diet resulted in rapid gain.

In the experiments with the Osborne-Mendel diet (four litters,

twenty-two rats) only the highest concentration of sulfur (1 per cent) was used. Table III presents a summary of the results with a typical litter. The retardation of growth was similar to that observed with the Sherman-Merrill diet but none of the animals died. Despite a satisfactory food intake, as shown in the table, the rats receiving sulfur failed to grow normally and the gain per 100 gm. of food was decidedly less than the controls. Although rats receiving both cystine and sulfur (Rats 215, 216) gained slightly more rapidly than the controls with neither added cystine nor sulfur, their rate of growth was much less than was

TABLE III.  
*Osborne-Mendel Diet.*

Period, 32 days.

Rat No.	Diet.	Initial weight.	Final weight.	Gain.	Total food.	Food per 100 gm. rat.	Gain per 100 gm. food.
		gm.	gm.	gm.	gm.	gm.	gm.
210	9% casein.	42.0	67.0	25.0	191.0	36.0	13.1
211	Same + 0.34% cystine.	35.0	85.0	50.0	191.5	32.1	26.0
212	“ + 0.34% “	32.0	72.0	40.0	158.5	32.7	25.2
213	“ + 1% sulfur.	52.0	65.5	13.5	149.0	29.5	9.1
214	“ + 1% “	45.0	56.5	11.5	179.5	40.7	6.4
215	“ + 0.34% cystine, 1% sulfur.	42.5	74.5	32.0	161.5	29.5	19.8
216	“ + 0.34% “ 1% “	42.5	67.5	25.0	147.5	30.6	16.9

to be expected in the presence of an adequate amount of cystine (*cf.* Rats 211, 212).

It remains to consider what was the immediate cause of death of the animals which died as a result of the administration of sulfur. While we have no direct evidence on this point, it seems probable that death was due to poisoning by hydrogen sulfide. Haibe (8) found that under conditions of chronic hydrogen sulfide poisoning, which is the condition with which we are concerned here, if the gas is being continually formed from sulfur in the intestine, the liver is the main organ affected. In many cases, we found hydrogen sulfide present in the abdominal cavity of rats to which sulfur had been fed. After the animal had died

from natural causes or had been killed with chloroform, the abdominal wall was exposed, a small incision made, and a strip of filter paper moistened with lead acetate was laid over the opening. In some cases, enough hydrogen sulfide was present to turn the paper a deep black, while in others, only a trace was detected. This was not a postmortem product because the number of positive tests was about equally divided between those animals examined some hours after death and those killed with chloroform and examined immediately. A darkening of the lead acetate was never obtained with the control rats which had never received sulfur in the diet.

The theory that the toxicity of the sulfur is due to the formation of hydrogen sulfide is confirmed by a consideration of the vast amount of pharmacological experimental data, which have been collected in connection with the therapeutic use of sulfur in various forms. Most investigators seem to consider that the hydrogen sulfide is a product of the action of intestinal bacteria (9). However, as shown by Heffter (10, 11) in the absence of bacteria, some proteins, especially egg white, and many animal tissues, including the intestinal mucosa, could form hydrogen sulfide from elementary sulfur. The formation of hydrogen sulfide in the intestine may be due to either or both reactions. Hydrogen sulfide is then absorbed, exerts its toxic action or is oxidized to sulfates (4).

It is difficult to explain the difference in toxicity of sulfur added to the Sherman-Merrill milk powder-starch diet and the Osborne-Mendel low protein diet. If the toxicity of the sulfur is due, as seems probable, to chronic hydrogen sulfide poisoning as a result of the formation of hydrogen sulfide by bacteria or by reaction with the intestinal mucosa, the lessened toxicity on the Osborne-Mendel diet might be explained by the higher fat content and slightly higher protein level. It seems possible that the fat might form a coating around the particles of sulfur and thus make more difficult the intimate contact of the sulfur with the intestinal mucosa or the action of bacteria. The higher protein level might also result in a better state of nutrition and hence a greater resistance to the toxic action. No simple explanation is evident for the apparent development of tolerance to the sulfur in the animals on the Sherman-Merrill diet, if they were able to survive

the first few weeks of the sulfur administration. Experiments which have for their purpose the explanation of the development of this tolerance are now in progress.

Bürgi and Gordonoff (12) have recently stated that the livers of rabbits fed sulfur for a long period had a glycogen content 2 to 3 times that of normal control animals. Details of their experiments have not to our knowledge been published. In view of their findings we have determined the glycogen content of the livers of a number of our experimental animals (Table IV). It

TABLE IV.

Diet.	Glycogen content of liver, per cent.								Average.
Basal (Sherman-Merrill).	1.73	6.20	3.93	1.32	0.91	0.83	1.00	2.27	
Basal + 0.5 per cent sulfur.	1.67	1.27	3.11	2.46	0.92				1.89
Basal + 0.75 per cent sulfur.	1.51	1.80	0.87	1.25	0.81	0.59	0.52	1.05	
Basal + 1 per cent sulfur.	1.35	1.30	0.75	0.63					1.01
Basal (Osborn-Mendel).	2.24	2.60	1.62						2.15
Basal + cystine (0.34 per cent).....	0.90	1.23	1.64	1.34					1.28
Basal + 1 per cent sulfur.	2.16	1.39	1.89	1.90	1.27	3.93			2.09
Basal + cystine and 1 per cent sulfur.....	1.02	0.86							0.94

will be seen that although there was a considerable variation in the percentage of glycogen present, no tendency was observed for the glycogen content of the livers of the rats to which sulfur was fed to be greater than those of the control animals. The findings of Bürgi and Gordonoff do not hold for rats under the conditions of our experiments. Unfortunately, details of their investigation are not available, and we are unable to compare their experiments with our own in respect to dosage of sulfur and duration of the experiments.

## SUMMARY.

Experiments are described in which flowers of sulfur in concentrations of 0.08, 0.50, 0.75, and 1.0 per cent were fed to young white rats maintained on two types of cystine-deficient basal diets (Sherman-Merrill milk powder-starch and Osborne-Mendel). No evidence was obtained to suggest that sulfur can replace cystine or in any way alter the cystine requirements for growth in the young white rat. When added to the milk powder-starch diet, sulfur was found to be definitely toxic and caused the death of fourteen out of twenty-two animals whose diet contained it to the amount of 1 per cent. After the addition of lower concentrations of sulfur to the milk powder-starch diet and the addition of 1 per cent sulfur to the Osborne-Mendel diet, marked retardation of the rate of growth was observed. The presence of amounts of cystine adequate to produce good growth did not affect the toxicity of the sulfur. The toxic effect was probably due to the formation of hydrogen sulfide in the intestine. There was no increase in the glycogen content of the livers of the rats to which sulfur had been fed, as compared with control animals.

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# THE SEPARATION OF LIPOID FRACTIONS FROM TUBERCLE BACILLI.\*

By R. J. ANDERSON.†

(From the Department of Chemistry, Yale University, New Haven.)

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## INTRODUCTION.

In connection with the chemical investigations of bacterial nucleic acid (1) and proteins (2) that have been conducted in this laboratory, an opportunity was presented for the examination of the lipid fractions obtained from a very large quantity of tubercle bacilli. The lipoids of this organism appear to possess more than ordinary biological and chemical importance not only from the point of view of the great resistance of the bacillus to destructive influences in the host (3) but also on account of the peculiar stimulus some of these components appear to exert on abnormal cell development in animals (4). Moreover specific antigenic properties have been attributed to certain alcohol-soluble lipid constituents of the bacillus (5).

In the hope of contributing some definite aid towards the solution of the complex problems involved in the study of tuberculosis a comprehensive cooperative research has been inaugurated dealing not only with the chemistry of the germ itself but also with the biological effects on animals of the various chemical compounds produced by this organism. The present report deals with methods of isolation of various lipid and carbohydrate fractions extracted from the living bacillus.

Much work has already been published by other investigators on this subject (6). All investigators have found that the bacilli

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† Holder of a Sterling Research Fellowship in chemistry at Yale University, 1926-27. This work has been supported in part by funds provided by the National Tuberculosis Association.



contain a high percentage of lipoids, varying from about 20 to 40 per cent. The variations in the amount of total lipoids that have been obtained depend apparently not only upon the age of the culture, the strain and virulence of the organism (7), but also upon the methods of extraction that have been employed (8). Many investigators have emphasized the difficulty of completely defatting the bacilli without first disrupting the cell membrane by treatment either with acids (Baudran (6)) or alkali (9).

A large number of constituents has been described as existing in the lipid fractions of tubercle bacilli. Earlier work on this subject has been reviewed in the comprehensive paper by Goris (10). Unfortunately much of this work was carried on with such small quantities of material that definite identification of many compounds would be impossible.

It would appear, however, that the lipoids consist mainly of a mixture containing wax and glycerides together with smaller quantities of phosphatides. In consequence of the high wax content, the total fat is peculiar in containing a very high percentage of unsaponifiable matter (11), the latter consisting largely of an alcohol called mycol by Tamura (6). The acid fastness of the bacillus has been ascribed to the presence of this alcohol (12).

The fact that the ether extract contained phosphorus led to the belief that the fat extracted from tubercle bacilli contained lecithin (13). A small quantity of a phosphatide preparation was isolated and analyzed by Tamura (6) who believed that the substance was a diamino monophosphatide. Agulhon and Frouin (6) on the other hand, described a preparation which after hydrolysis gave fatty acids, glycerol, a base analogous to choline, and a gummy substance which liberated glucose by acid hydrolysis. These authors concluded, therefore, that the phosphatide was not a definite chemical entity but a mixture analogous to jecorin. It has been claimed by Koganei (14) that the fatty substances of tubercle bacilli contain phrenosin, kersasin, sphingomyelin and cephalin but these claims appear to rest upon extremely slight foundations.

In addition to proteins and lipoids of biological importance it is evident from the experiments of Laidlaw and Dudley (15) and of Mueller (16) that tubercle bacilli produce polysaccharides that possess specific properties. In this connection it may be of inter-

est to notice that we succeeded in separating a notable quantity of a polysaccharide from the alcohol-ether extract of the bacilli. The product was obtained in the form of a non-hygroscopic nearly white powder.

For our experiments we were anxious to secure the lipoid material as similar as possible to the condition in which it functioned in the living cells. It was necessary therefore to guard against changes induced either by heat or by oxidation. To achieve our objective moist, living bacilli were extracted at room temperature with a mixture of alcohol and ether followed by extraction with chloroform. A rigid exclusion of air was maintained throughout all of the various manipulations by using an atmosphere of carbon dioxide.

All solvents that were used had been purified by distillation and the alcohol had been distilled over potassium hydroxide. The purified solvents were saturated with carbon dioxide and the air was displaced by the same gas from all vessels before any material was introduced into them. During filtrations streams of carbon dioxide were passed over the Buchner funnels.

The voluminous extracts were concentrated under reduced pressure at a temperature which was not allowed to exceed 35°C. and carbon dioxide was admitted through the capillary tube during the distillation.

Through the cooperation of the Research Committee of the National Tuberculosis Association and the H. K. Mulford Company, a very large quantity of living bacilli was provided for this work. This material was extracted as described in the "Experimental Part" and the precautions for exclusion of air, as mentioned above, were carefully observed. The fresh moist bacteria were first extracted with a mixture of alcohol and ether. The resulting extract contained mostly glycerides and phosphatides and only a small quantity of wax. These constituents could be separated without much difficulty into the three groups mentioned above from ethereal solution by means of acetone. In addition to lipoids the alcohol-ether extract contained some basic compounds that could be precipitated by mercuric chloride and by phosphotungstic acid and also a considerable amount of a polysaccharide. The latter substance in the pure state is quite insoluble in alcohol and ether but it was present in the extract

probably by reason of the moisture contributed by the fresh bacteria to the alcohol-ether mixture. The polysaccharide could be precipitated from aqueous solution by alkaline basic lead acetate.

The bacterial residue from the alcohol-ether extraction was twice digested in chloroform. On evaporation of the solvent a large quantity of crude wax was obtained.

It was possible therefore by this procedure to separate the lipid material into three fractions consisting of glycerides, phosphatides, and wax. Such a preliminary fractionation is of great advantage in subsequent chemical and biological examinations. Quantitatively the lipoids are present in the following proportions: wax, glycerides, phosphatides, the wax constituting more than one-half of the total lipoids.

The lipid material contained a surprisingly large amount of phosphatides. This fraction has been examined more fully than any of the others both chemically and biologically. The biological investigations of this substance carried out in Dr. Sabin's laboratory at the Rockefeller Institute for Medical Research and in Dr. Chamber's laboratory at Cornell University Medical College, have revealed very interesting and important properties. These observations will be published shortly from the respective laboratories.

The results of the chemical investigations of this fraction will be reported in a separate paper. As soon as time will permit the various other fractions will also be subjected to chemical and biological investigations.

#### EXPERIMENTAL PART.

The human type of tubercle bacilli, Strain H-37, was used. The bacteria were grown in the laboratory of H. K. Mulford Company, on the Long synthetic medium (17). The cultures, each representing about 200 cc. of the medium, were contained in 1 liter bottles and they were incubated for a period of 6 weeks. For the present work 2200 cultures were provided.

*Extraction with Alcohol and Ether.*—The bacilli were collected on large Buchner funnels and washed with water. The moist material was immediately placed in a mixture consisting of equal parts of 95 per cent alcohol and U.S.P. ether. The solvent was contained

in 5 gallon Pyrex bottles and the bacilli from about 450 cultures were introduced into each bottle. The containers were securely closed with cork stoppers and transported to the Sterling Chemistry Laboratory at New Haven.

The contents of the bottles were agitated frequently for about 4 weeks and were then allowed to settle. The clear slightly amber-colored supernatant liquid was syphoned off and the nearly white bacterial residue was filtered on Buchner funnels and washed with ether. The alcohol-ether extract obtained in this manner was concentrated and worked up as will be described later.

*Extraction of the Bacterial Residue with Chloroform.*—The bacterial residue from the alcohol-ether extraction was immediately transferred into a 5 gallon bottle and digested in chloroform with occasional stirring for about 4 weeks. The material was then filtered on Buchner funnels, washed with chloroform, and the residue was subjected to a second extraction with chloroform for 1 week.

The insoluble bacterial residue was then filtered off, washed with chloroform and dried. It formed a light gray powder that weighed 2902 gm. This material was preserved for further investigations of nucleic acids, proteins, and carbohydrates. It was designated as Fraction A-10.

The chloroform extracts and washings were concentrated by distillation and the residue was dried in a current of carbon dioxide. The material formed a light yellow-colored wax. It was designated Fraction A-6 and it weighed 427 gm.

*Examination of the Alcohol-Ether Extract.*—The alcohol-ether extract formed a perfectly clear, pale straw-colored liquid. The ether was distilled off in a current of carbon dioxide and the alcohol was distilled under reduced pressure. After most of the alcohol had been removed, a residue of lipid material remained suspended in the dilute aqueous alcohol. This mixture was repeatedly shaken with ether in separatory funnels until the lipoids were extracted.

The dilute aqueous alcoholic solution which remained after the ether extraction was reserved for a special examination as will be described later.

*Separation of the Ether-Soluble Lipoids into Acetone-Soluble and Acetone-Insoluble Fractions.*—The ethereal extract, obtained as

mentioned above, was dried with anhydrous sodium sulfate, filtered, and concentrated to a volume of about 500 cc. and an equal volume of acetone was added when a dense gummy precipitate separated which formed a sticky mass on the bottom of the flask. The clear supernatant liquid was decanted and the precipitate was washed by stirring thoroughly with several portions of acetone. The washings were united with the mother liquor; the solution was again concentrated and mixed with more acetone when a further quantity of the gummy material was precipitated which was increased by cooling the mixture with ice water. The supernatant liquid was decanted and the residue, after it had been washed with acetone, was united with the first acetone precipitate. This material which had been precipitated by acetone consisted of crude phosphatide and wax and it was purified as will be described later.

The last mother liquor and washings were concentrated by distillation and the residue was dried in a current of carbon dioxide. The substance was a dark brown oil that formed a soft fatty mass on cooling to room temperature. This fraction which represents the acetone-soluble fat weighed 240 gm. It was designated Fraction A-5 and it was reserved for a complete chemical examination.

*Purification of the Crude Phosphatide.*—The acetone-insoluble fraction was dissolved in 200 cc. of ether in which it gave a nearly clear amber-colored solution. It was again precipitated by adding an equal volume of acetone. The substance was reprecipitated in this manner five times but the last precipitation was made by pouring the ethereal solution into 750 cc. of ice-cold acetone when the insoluble material separated in the form of a faintly yellowish granular powder. The substance was collected on a Buchner funnel, washed with acetone, and dried in a vacuum desiccator over sulfuric acid and carbon dioxide. The dried substance was a nearly white non-hygroscopic powder that weighed 148.5 gm. It represents the first crude phosphatide fraction and it was designated Fraction A-3.

For analysis the substance was dried at 56°C. *in vacuo* over phosphorus pentoxide but the loss in weight was very slight.

0.3789 gm. substance: 0.0362 gm.  $Mg_2P_2O_7$ .

0.4478 " " required (Kjeldahl) 1.33 cc. 0.1 N HCl.

Found. P 2.66, N 0.41 per cent.

The ether-acetone mother liquors, which remained after the above mentioned precipitations, were cooled for some time in a freezing mixture of ice and salt. A large quantity of a nearly white precipitate separated. The substance was filtered on a Buchner funnel and washed with acetone.

The mother liquor and washings were concentrated by distillation until the ether was removed. The solution was cooled in a freezing mixture when a further quantity of a white precipitate was obtained. The latter was filtered off, washed with cold acetone and united with the similar product mentioned above, and dried in the manner already described. The substance formed a nearly white non-hygroscopic powder that weighed 104.6 gm., and which was designated Fraction A-4.

The final mother liquor was concentrated and the residue was dried in a current of carbon dioxide. The slight amount of fat that remained was united with the first portion of acetone-soluble fat.

Analysis of Fraction A-4 showed that it contained only 0.86 per cent of phosphorus. It is probable therefore that this product contained a large proportion of wax.

*Examination of the Aqueous-Alcohol Solution.*—The aqueous-alcohol solution that remained after the lipoid substances had been extracted with ether measured about 12 liters. It was light yellow in color and showed an acid reaction to litmus. The solution was precipitated by adding in slight excess a 20 per cent solution of lead acetate. This precipitate was filtered off and discarded. The filtrate was freed from lead by hydrogen sulfide, filtered, and concentrated by distillation under reduced pressure to a volume of about 400 cc. The solution was mixed with 10 per cent mercuric chloride in 95 per cent alcohol so long as any precipitate was produced. The precipitate was centrifuged, washed with water, suspended in water, and decomposed by hydrogen sulfide. The mercuric sulfide was filtered off and the filtrate was concentrated *in vacuo* and finally dried in a vacuum desiccator over sulfuric acid. A semicrystalline substance remained that weighed 7.2 gm. and it was designated Fraction A-7.

The mother liquor from the mercuric chloride precipitate was freed from excess of mercury by hydrogen sulfide. The precipitate was filtered off and the filtrate was concentrated *in vacuo* to

a volume of about 500 cc. The solution was acidified until it contained 5 per cent of sulfuric acid and phosphotungstic acid containing 5 per cent sulfuric acid was added until no further precipitate was produced.

The phosphotungstate was collected on a Buchner funnel, washed with 5 per cent sulfuric acid, and decomposed with an excess of barium hydroxide. The barium precipitate was filtered and thoroughly washed with water. The filtrate was freed from excess of barium quantitatively by sulfuric acid and after the barium sulfate had been removed the solution was concentrated *in vacuo*. The syrupy residue which was strongly alkaline in reaction was neutralized with hydrochloric acid and finally evaporated to dryness in a vacuum desiccator over sulfuric acid. The substance which was semicrystalline weighed 5.3 gm. and it was designated Fraction A-9.

*Isolation of the Polysaccharide.*—Barium hydroxide was added in slight excess to the filtrate from the phosphotungstic acid precipitate and the barium phosphotungstate and sulfate were filtered off and washed with water. The excess of barium in the filtrate was removed quantitatively with sulfuric acid. After the barium sulfate had been removed, the filtrate was concentrated *in vacuo* to a volume of about 200 cc. Lead acetate was then added in slight excess and the lead chloride that separated was filtered off and basic lead acetate was added until no further precipitate was produced. The mixture was made strongly alkaline with ammonium hydroxide and allowed to stand overnight. The white precipitate was collected on a Buchner funnel, thoroughly washed with water, suspended in water, and decomposed with hydrogen sulfide. After removing the lead sulfide, the filtrate was concentrated *in vacuo* and finally dried to a thick syrup in a vacuum desiccator.

The material was extremely soluble in water but only slightly soluble in alcohol. It showed no tendency to crystallize. The aqueous solution gave no reduction on boiling with Fehling's solution and even after heating for some time with dilute hydrochloric acid no reduction was obtained. After the substance had been boiled for 5 minutes with dilute hydrochloric acid, however, it gave a heavy reduction with Fehling's solution.

In order to obtain the sugar complex in solid form the above

mentioned thick syrup was ground up in a mortar under a large quantity of absolute alcohol. The syrup was gradually converted into a nearly white powder by continuing the grinding until the water which was present had been absorbed by the absolute alcohol. The powder was filtered off on a Buchner funnel and washed with absolute alcohol. After drying in a vacuum over sulfuric acid the substance was obtained as a heavy nearly white powder that weighed 33.9 gm. This material which evidently represents a stable polysaccharide was designated as Fraction A-8.

The substance was acid in reaction and it contained traces of phosphorus, nitrogen, and sulfur. The acidity and phosphorus

TABLE I.

*Lipoid Fractions and Other Compounds Separated from 2000 Cultures of Tubercle Bacilli.*

Fraction.	No.	Weight.
		<i>gm.</i>
First phosphatide. ....	A-3	148.5
Second " .....	A-4	104.6
Acetone-soluble fat.....	A-5	240.0
Chloroform-soluble wax.....	A-6	427.0
Base precipitated by HgCl <sub>2</sub> .....	A-7	7.2
" " " phosphotungstic acid.....	A-9	5.3
Polysaccharide. ....	A-8	33.9
Dried bacterial residue.....	A-10	2902.0
Total weight.....		3868.5

content may have been due to the presence of a small amount of phosphoric acid. The quantity of nitrogen present appeared to be very small. Lack of time has prevented any further chemical investigation of this interesting compound but preliminary biological experiments are now under way.

In Table I are summarized the various fractions that were isolated from the alcohol-ether and chloroform extracts of 2000 cultures of fresh tubercle bacilli. From the data given in the table it is evident that each culture contained on an average somewhat less than 2 gm. of dry bacteria and the total lipid material represented 23.78 per cent of the dry bacilli.



In conclusion we acknowledge with pleasure our indebtedness to Dr. Wm. Charles White, Chairman of the Research Committee of the National Tuberculosis Association, to H. K. Mulford Company, and to Professor T. B. Johnson whose cooperation made this work possible.

#### SUMMARY.

The lipid constituents of tubercle bacilli have been examined. Methods are described for the separation of this material into three groups consisting of wax, glycerides, and phosphatides. In addition to the lipoids, alcohol-ether extracts of fresh tubercle bacilli contain basic substances and a notable quantity of a polysaccharide.

The bacilli contain a surprisingly large amount of phosphatides and this fraction possesses unusually important biological properties.

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# A STUDY OF THE PHOSPHATIDE FRACTION OF TUBERCLE BACILLI.\*

By R. J. ANDERSON.†

(From the Department of Chemistry, Yale University, New Haven.)

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## INTRODUCTION.

In a previous report from this laboratory (1) we have described the separation of the lipid material from tubercle bacilli into three major groups consisting of wax, glycerides, and phosphatides. The amount of phosphatide<sup>1</sup> that was isolated from the alcohol-ether extract of the bacilli was surprisingly large. This heretofore little known material has been examined and the present paper deals mainly with a study of its composition and cleavage products.

Hammerschlag (2) who first examined the lipoids from tubercle bacilli noted that the ether extract contained phosphorus and concluded therefore that the fat contained lecithin. Similar observations were recorded by Kresling (3), Baudran (4), Auclair and Paris (5), Aronson (6), Bürger (7), and by Goris (8). Tamura (9) described a phosphatide preparation obtained from an alcoholic extract of tubercle bacilli which he regarded as a diaminomono-phosphatide. Agulhon and Frouin (10) showed that the phosphatide which they isolated contained glucose and these authors

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† Holder of a Sterling Research Fellowship in chemistry at Yale University, 1926-27. This work has been supported in part by funds provided by the National Tuberculosis Association.

<sup>1</sup> In the abstract given before the American Society of Biological Chemists this substance was referred to under the name of phosphosucride. It was stated at that time that glycerol had not been detected among the cleavage products but we have since discovered that the water-soluble constituents obtained after hydrolyzing the phosphatide contain about 5 per cent of unchanged glycerophosphoric acid.

concluded that the material was a mixture similar to jecorin. Dienes and Schoenheit (11) obtained preparations that contained phosphorus, nitrogen, and varying amounts of glucose. It has been claimed by Koganei (12) that substances such as phrenosin, kersin, sphingomyelin, and cephalin occur in the fat from tubercle bacilli but no definite proof was given that these compounds were present.

It would appear therefore that no definite knowledge exists at the present time regarding the nature and properties of the phosphatide of tubercle bacilli.

The preparations designated as Fraction A-3 and A-4 in the publication (1) already referred to, which contained relatively large amounts of phosphorus, have been studied. Both fractions were first reprecipitated several times from ethereal solution by acetone. The composition of Fraction A-3 remained unaltered but a considerable amount of wax was eliminated from Fraction A-4 by this treatment. The composition of the purified top fraction of No. A-4 was practically identical with that of No. A-3.

The total weight of the crude phosphatide fractions was 253.1 gm. The wax that was removed from Fraction A-4 weighed 58.6 gm. The purified phosphatide amounted therefore to 194.5 gm., which is somewhat more than 5 per cent of the dry bacilli.

The phosphatide fractions of tubercle bacilli appear to possess unusually interesting and important biological properties. This phase of the subject is being investigated in Dr. Sabin's laboratory at the Rockefeller Institute for Medical Research and in Dr. Chamber's laboratory at Cornell University Medical College.

#### EXPERIMENTAL PART.

*Purification of Fraction A-4.*—During the operations described below every effort was made to exclude air by using an atmosphere of carbon dioxide. Redistilled anhydrous ether and redistilled acetone were employed and these solvents were saturated with carbon dioxide. The air was displaced by carbon dioxide from all vessels and desiccators before any preparations were introduced.

The substance which weighed 104.6 gm., was dissolved in 300 cc. of ether in which it formed a nearly colorless solution. The addition of an equal volume of acetone caused a precipitate which formed an oily layer on the bottom of the flask. The liquid layer

solidified on cooling for a few minutes in ice water and the supernatant liquor was decanted. These precipitations were repeated twice from 200 cc. of ether and seven times from 100 cc. of ether. The last precipitation was made by pouring the ethereal solution into 350 cc. of ice-cold acetone when the substance separated as a nearly white granular powder. The precipitate was filtered off on a Buchner funnel, washed with acetone, and dried in a vacuum desiccator over sulfuric acid. The dried substance weighed 41.5 gm. On analysis it was found to contain 1.98 per cent of phosphorus.

The mother liquors were concentrated by distillation until the ether was removed. On cooling a solid wax-like mass separated. The supernatant liquid was decanted, concentrated to about 100 cc., and cooled in ice water when an amorphous substance separated which was filtered off and united with the wax-like precipitate. The total material was now dissolved in 200 cc. of ether and the solution was poured into 750 cc. of acetone that had been cooled to  $-5^{\circ}\text{C}$ . The white amorphous precipitate which separated was filtered on a Buchner funnel, washed with acetone, and dried *in vacuo*. This fraction weighed 58.6 gm. Heated in a capillary tube the substance softened at  $41^{\circ}\text{C}$ . and melted at  $45-47^{\circ}\text{C}$ . On analysis it was found to contain only 0.3 per cent of phosphorus and 0.07 per cent of nitrogen. This substance was therefore nearly free from phosphatide and consisted apparently of wax.

The top fraction mentioned above was again precipitated ten times from its solution in 100 cc. of ether by adding an equal volume of acetone. The last precipitation was made, however, by pouring the ethereal solution with constant stirring into 500 cc. of ice-cold acetone. When the substance is precipitated in this manner it separates in the form of a nearly white granular powder but when the acetone is added to the ethereal solution a gummy precipitate is produced. The material was filtered, washed with acetone, and dried in a vacuum desiccator over sulfuric acid. The dried substance weighed 38 gm. The recovery was therefore nearly quantitative.

For analysis the substance was dried at  $56^{\circ}\text{C}$ . in a vacuum desiccator over phosphorus pentoxide. Found, P 2.14, N 0.40 per cent.

The results show that the last ten precipitations had caused but little change in composition. The percentage of phosphorus and of nitrogen in the top fraction after twenty precipitations were practically the same as in Fraction A-3. These fractions are therefore very likely identical.

*Purification of Phosphatide Fraction A-3.*—115 gm. of Fraction A-3 were dissolved in 200 cc. of ether and the solution was mixed with an equal volume of acetone. The dense gummy precipitate collected immediately on the bottom of the flask leaving a clear supernatant liquid which was decanted. The precipitate was dissolved in 130 cc. of ether and mixed with an equal volume of acetone. These operations were repeated ten times but the last precipitation was made by pouring the ethereal solution into 750 cc. of ice-cold acetone. The precipitate which was then produced was filtered, washed with acetone, and dried as mentioned above. The product was a nearly white, granular non-hygroscopic powder and it weighed 100.2 gm.

The mother liquors were concentrated to dryness in a stream of carbon dioxide and the residue was dried *in vacuo* over sulfuric acid. It formed a yellowish somewhat sticky mass that weighed 13.3 gm.

For analysis the samples were dried at 56°C. in a vacuum over phosphorus pentoxide. The loss in weight was very slight.

Found.	Top fraction of No. A-3.	P 2.30, N 0.36 per cent.
	Bottom " " " "	" 2.03, " 0.35 " "

The results indicate that reprecipitation had caused no appreciable change in composition. While there was no change in composition it cannot be claimed that the compound represents a chemically homogeneous substance. It is only too well known that amorphous products and especially phosphatides are purified with great difficulty.

The top of Fraction A-3 possessed the following properties: Heated in a capillary tube the substance darkened at about 200° and melted to a dark brown liquid at 210°. It was easily soluble in ether, chloroform, or benzene, and it was precipitated almost quantitatively by adding acetone, alcohol, or methyl alcohol. When rubbed up with water it formed an opalescent solution which

was precipitated by the addition of acids or salts. The aqueous suspension gave no reduction when boiled with Fehling's solution.

When an aqueous suspension of the phosphatide was strongly acidified with hydrochloric acid a heavy precipitate was formed. When this mixture was boiled for several minutes the coagulum gradually was converted into an oily layer. The oil solidified on cooling. The acid solution was filtered, neutralized with sodium hydroxide, and boiled with Fehling's solution when a heavy reduction occurred. The results indicate that a carbohydrate complex is combined in some manner in the phosphatide molecule. Since the substance only reduced Fehling's solution after hydrolysis it appears probable that the sugar exists in some chemical combination.

*Hydrolysis of the Top Fraction of No. A-3.*—Without subjecting the phosphatide to any further so called purification processes, it was hydrolyzed and a study was made of its cleavage products.

Throughout the operations described below air was excluded as much as possible by using an atmosphere of carbon dioxide, the latter displacing the air in vacuum desiccators before drying the fatty acids. The ether that was used had been saturated with carbon dioxide and a stream of this gas was passed through the apparatus during hydrolysis and also during the concentrations of ethereal solutions.

In a preliminary experiment 4.9435 gm. of dry phosphatide were hydrolyzed by refluxing with 5 per cent sulfuric acid until the insoluble matter had been converted into a clear oily layer that floated on top of the dilute acid. The time required was 7 hours.

The fatty acids, which formed a solid cake on cooling, were extracted with ether. The ethereal solution was washed with water, filtered, and evaporated to dryness. The residue was dried in a vacuum desiccator over sulfuric acid. It formed a light brown cake that weighed 3.2955 gm., corresponding to 66.65 per cent of fatty acids.

*Examination of the Dilute Acid Solution. a. Quantitative Determination of the Reducing Sugar.*—The ether that remained in the aqueous liquid was removed by distillation. The solution was cooled and made up to 250 cc. Reducing sugars were determined in 25 cc. portions by Fehling's solution. Found: cuprous



oxide, 0.1518 gm. and 0.1534 gm., corresponding to 13.96 per cent of glucose.

*b. Preparation of the Osazone.*—Another portion of 50 cc. of the above solution was neutralized with sodium hydroxide and heated on the water bath with phenylhydrazine hydrochloride and sodium acetate. Crystals of the osazone began to separate after the solution had been heated for 15 minutes. After 1 hour the solution was cooled and the crystals were filtered off and washed with water. The crystals were dissolved in hot alcohol, diluted with an equal volume of water, boiled with norit, and filtered. As the solution cooled delicate yellow needles separated which appeared to be identical with glucosazone crystals.

In order to identify this osazone a sample of glucosazone was prepared from commercial dextrose and it was purified in the manner described above. The two products appeared to be identical. When heated rapidly in capillary tubes both osazones melted with decomposition at 208°C. (uncorrected) and a mixture of the two preparations also decomposed at the same temperature.

The optical rotation of 0.1 gm. of the osazones dissolved in 4 cc. of pyridine and 6 cc. of absolute alcohol as described by Neuberg (13) was determined in a 1 dm. tube. The osazone from the phosphatide sugar gave a reading of  $-0.64^{\circ}$  and that of the glucosazone was  $-0.65^{\circ}$ . It is evident therefore that the reducing sugar that is formed on hydrolyzing the phosphatide is glucose.

*c. Examination of Aqueous Solution for Glycerol and Base.*—A qualitative test showed that the dilute acid solution contained a large quantity of phosphoric acid. It was assumed therefore that the glycerophosphoric acid, if present originally, had been hydrolyzed. The aqueous solution should also contain choline if this substance was present in the phosphatide.

The balance of the aqueous solution was therefore freed from phosphoric and sulfuric acids by barium hydroxide and the excess of the latter was removed quantitatively by sulfuric acid. The filtered solution was evaporated to dryness *in vacuo* and the residue was extracted with several portions of warm absolute alcohol. The addition of an alcoholic solution of platinum chloride caused no precipitate, which indicated that choline was absent.

The alcohol was evaporated and the residue which formed a thick syrup was dried in a vacuum desiccator over sulfuric acid.

This residue, after fusing with sodium gave no reaction for nitrogen. Evidently, therefore, the substance did not contain choline or any other analogous base. The small amount of nitrogen present in the phosphatide might have been in the form of ammonia and the latter had been lost during the concentration of the aqueous solution.

The syrupy residue gave no reaction for glycerol and it was assumed at first that the phosphatide did not contain glycerol. Later on it was discovered that while glycerol could not be detected, the aqueous solution contained a large percentage of glycerophosphoric acid.

That glycerophosphoric acid is not hydrolyzed by boiling for 7 or even 11 hours with 5 per cent sulfuric acid is not surprising in view of the data published by Malengreau and Prigent (14).

*Further Hydrolysis of the Phosphatide Fraction A-3.*—In order to study the water-soluble constituents more closely 14.8904 gm. of the same Fraction A-3 were hydrolyzed by boiling with 5 per cent sulfuric acid for 10 hours and the cleavage products were separated as described above. The fatty acids weighed 9.9357 gm. corresponding to 66.73 per cent.

The dilute acid solution was made up to 1000 cc. and it was examined as follows: (a) The reducing sugar determined by Fehling's solution corresponded to 13.39 per cent of glucose. (b) 250 cc. of the solution were made alkaline with sodium hydroxide and distilled into 0.1 N hydrochloric acid. Required 5.95 cc. of 0.1 N HCl.  $\text{NH}_3 = 0.27$  per cent. (c) The balance of the aqueous solution, 700 cc., was freed from phosphoric and sulfuric acids by a slight excess of barium hydroxide, filtered, and the filtrate was distilled under reduced pressure until the ammonia was removed. The excess of barium was then precipitated quantitatively by sulfuric acid. The solution, after it had been filtered, was concentrated *in vacua* to a small volume and made up to 50 cc. In a 2 dm. tube this solution showed a dextrorotation of  $+0.93^\circ$ .

5 cc. of the solution were dried to constant weight in a vacuum desiccator over sulfuric acid. The residue weighed 0.3455 gm., consequently the original 1000 cc. should have contained 4.9357 gm., which is equal to 33.14 per cent of the phosphatide. It is evident from this result that in addition to glucose the solution contained a large amount of some other substance. It was dis-

covered that two additional compounds were present, *viz.* glycerophosphoric acid and some other acid, probably a sugar acid, which gave a very sparingly soluble crystalline compound with phenylhydrazine.

The three constituents mentioned above were separated as follows:

*a. Isolation of Barium Glycerophosphate.*—The aqueous solution was concentrated in a vacuum desiccator until a thick syrup remained. The syrup was extracted with several portions of hot absolute alcohol. A few drops of phenolphthalein were added to the alcoholic solution and it was titrated to neutrality with saturated aqueous barium hydroxide. A heavy precipitate of barium glycerophosphate separated and was filtered off, washed with alcohol, and dried. The mother liquors were saved and examined as described later on. The crude barium glycerophosphate was dissolved in water and precipitated by adding alcohol. These operations were repeated three times. The barium salt was a snow-white amorphous powder. For analysis it was dried at 105°C. in a vacuum over phosphorus pentoxide.

0.2453 gm. substance lost on drying 0.0169 gm. or 6.88 per cent.

0.2284 " " : 0.1678 gm. BaSO<sub>4</sub> and 0.0753 gm. Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

Calculated. C<sub>3</sub>H<sub>7</sub>O<sub>6</sub>PBa +  $\frac{1}{2}$  H<sub>2</sub>O (mol. wt. 316). Ba 43.35, P 9.81 per cent.

Found. " 43.29, " 9.20 " "

The analytical values agree with the calculated composition of barium glycerophosphate plus one-half molecule of water. A sample of the air-dried salt was analyzed with the following result.

0.2056 gm. substance: 0.1414 gm. BaSO<sub>4</sub> and 0.0638 gm. Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

Calculated. C<sub>3</sub>H<sub>7</sub>O<sub>6</sub>PBa + 2H<sub>2</sub>O (mol. wt. 343). Ba 39.94, P 9.03 per cent.

Found. " 40.07, " 8.64 " "

*b. Separation of the Sugar Acid from Glucose.*—The alcoholic mother liquors mentioned above were concentrated to dryness. The residue was dissolved in water and the barium was precipitated quantitatively with sulfuric acid. The solution was filtered and concentrated to a syrup *in vacuo*. The syrup was dissolved in a little water and united with the alcohol-insoluble portion of the original syrup. A slight excess of phenylhydrazine, dissolved in a little alcohol, was added. Almost immediately nearly colorless

crystals began to separate. After the mixture had stood at room temperature for 2 hours and 1 hour in ice water, the crystals were filtered off, washed with water, and dried. Heated in a capillary tube the substance melted with decomposition at 194–195°C. (uncorrected). The crystals were almost insoluble in alcohol and very slightly soluble in hot water. Continued boiling was necessary to bring the substance into solution but the color turned yellow and on cooling brownish yellow crystals separated. Lack of time has prevented the identification of this substance.

*c. Separation of Glucose as Glucosazone.*—The mother liquor from the above mentioned crystals was mixed with phenylhydrazine hydrochloride and sodium acetate and the solution was heated on the water bath. Typical glucosazone crystals began to separate after the solution had been heated for 15 minutes. After the osazone had been purified as already described, it melted with decomposition at 208°C. (uncorrected).

The procedure described above permits of the separation of the three water-soluble constituents; *viz.*, glycerophosphoric acid, the unidentified substance giving a crystalline compound with phenylhydrazine, and glucose.

*Determination of Ammonia in the Phosphatide.*—The results described on p. 543 would indicate that the dilute acid solution after hydrolyzing the phosphatide contained practically all of the nitrogen as ammonia. The following determinations of ammonia were made by distilling a mixture of the phosphatide with dilute alkali.

In the first experiment 5.3373 gm. of phosphatide, 300 cc. of water, 50 cc. of concentrated sodium hydroxide, and 10 gm. of barium hydroxide were distilled into 0.1 N hydrochloric acid. Required 8.97 cc. 0.1 N HCl.  $\text{NH}_3 = 0.28$  per cent.

In a second experiment 3.0211 gm. of phosphatide, 300 cc. of water, 5 cc. of concentrated sodium hydroxide, and 10 gm. of barium hydroxide were distilled. Required 4.59 cc. of 0.1 N HCl.  $\text{NH}_3 = 0.25$  per cent.

The results show that practically all of the nitrogen that is contained in the phosphatide can be distilled off in the form of ammonia in the presence of dilute alkali. Since the phosphatide contained only about 0.3 per cent of nitrogen it appears very probable that ammonia is the only nitrogenous base present and it is probably bound on the phosphoric acid.

*Examination of the Fatty Acids Liberated on Hydrolyzing the Phosphatide.*—It has been indicated earlier that the fatty acids amounted to 66 to 67 per cent of the phosphatide. At room temperature the mixture formed a light brown crystalline mass. The iodine number was low, varying from 18.6 to 18.7. The acids were converted into the lead soaps; the latter were dried and extracted with ether. These fractions were examined as described below.

*Ether-Insoluble Lead Soap.*—The ether-insoluble lead salt was decomposed with dilute hydrochloric acid. The fatty acids were extracted with ether. The ethereal solution was washed with water, filtered, and the ether was evaporated, leaving a colorless crystalline mass. The substance was recrystallized three times from alcohol and five times from acetone. The snow-white crystals melted at 62°C. (uncorrected).

The following values were obtained on titrating the alcoholic solution of the acid with alcoholic potassium hydroxide using phenolphthalein as indicator.

0.5179 gm. substance required 19.94 cc. 0.1 N KOH.

0.4388 " " " 16.98 " 0.1 " "

Found. Mol. wt. 259, 258.

The melting point and molecular weight indicate that the acid was palmitic acid which melts at 62–63°C. and the molecular weight is 256.

*Ether-Soluble Lead Soaps.*—The ethereal solution containing the soluble lead salts was shaken with dilute hydrochloric acid and the lead chloride was removed. After washing the ethereal solution several times with water it was filtered and the ether was distilled. The residue which was a slightly brownish oil was dried in a vacuum desiccator over sulfuric acid. The iodine number, determined by the Hanus method, varied in different lots from 30.8 to 31.1 which indicated the presence of a large proportion of a liquid saturated acid.

*Reduction of the Unsaturated Acid.*—The liquid fatty acids, 7.2 gm., were dissolved in alcohol and 0.2 gm. of platinum oxide (15) was mixed with the solution. The flask containing the mixture was evacuated, hydrogen was introduced, and the apparatus was shaken for 2 hours under a pressure of about 1 pound of hydrogen.

The reaction mixture was then shaken with air until the catalyst was precipitated. The solution was filtered and cooled in ice and salt when colorless crystals separated. The crystals were filtered off, washed with ice-cold alcohol, and dried. The filtrate was concentrated to dryness and the oily residue was dissolved in a little petroleum ether and again cooled in a freezing mixture when a further quantity of crystals was obtained which was collected and added to the first lot.

It was impossible however to remove all of the reduced acid by crystallization. To accomplish a more complete separation the liquid portion of the fatty acids was converted into the lead salt and the latter was extracted with ether. The ether-insoluble lead salt was decomposed with hydrochloric acid and extracted with ether. On evaporation of the solvent a further quantity of colorless crystals was obtained.

The total amount of the crystalline acid obtained in this manner was 2.65 gm. This product was dissolved in alcohol, treated with norit, and recrystallized five times from alcohol. The snow-white crystals melted at 69–70°C. (uncorrected). On titration the following result was obtained:

0.2884 gm. substance required 10.23 cc. 0.1 N KOH.

Found. Mol. wt. 281.

The melting point and molecular weight prove that the reduced acid was stearic acid which melts at 69°C., and possesses the molecular weight of 284.

The original mixture of saturated and unsaturated fatty acids, weighing 7.2 gm., had an iodine number of 31. Hence it should have contained 34 per cent or 2.45 gm. of oleic acid. The amount of crude stearic acid obtained after reduction as shown above, was 2.65 gm. The results indicate that the unsaturated acid contained in the phosphatide was oleic acid.

*Isolation of the Liquid Saturated Fatty Acid.*—The ether-soluble lead salt, after the reduced acid had been removed, was decomposed with dilute hydrochloric acid and the liquid saturated fatty acid was isolated in the manner described above. It formed a faintly yellow oil that weighed 3.8 gm. In chloroform solution there was no evidence of absorption of bromine, indicating that the acid was saturated. It was miscible in all proportions with

organic solvents. The acid solidified when cooled in ice water but at room temperature it was an odorless oil.

The following results were obtained on titration with 0.1 N alcoholic potassium hydroxide.

0.2644 gm. acid required 8.54 cc. 0.1 N KOH.

0.7312 " " " 23.30 " 0.1 " "

Found. Mol. wt. 309, 313. Average mol. wt. 311.

The acid was optically active.

1.1193 gm. acid dissolved in alcohol and made up to 18 cc.

In a 2 dm. tube  $\alpha = +0.207^\circ$ ;  $[\alpha]_D^{20} = +1.66^\circ$ .

The high molecular weight, the optical activity, and the fact that the substance is a liquid at ordinary temperature would indicate that the acid must possess an unusual chemical constitution. On analysis the following values were found.

0.1556 gm. substance 0.1791 gm.  $H_2O$  and 0.4346 gm.  $CO_2$ .

0.1151 " " 0.1321 " " " 0.3217 " "

Found. C 76.17, 76.22, H 12.88, 12.84 per cent.

The values for carbon are undoubtedly slightly low because traces of carbon remained on the tube and boat. The results show nevertheless that the acid must have a high molecular weight.

*Preparation of a Crystalline Salt of the Liquid Saturated Acid with Benzyl Pseudothiourea.*—Benzyl pseudothiourea possesses the property of yielding readily crystalline salts with all fatty acids.<sup>2</sup> The calculated quantity of benzyl pseudothiourea hydrochloride was dissolved in alcohol and titrated to neutral reaction with an alcoholic solution of potassium hydroxide, phenolphthalein being used as indicator. The potassium chloride was filtered off and washed with a little absolute alcohol. On mixing the solution of the free thiourea with a concentrated alcoholic solution of the liquid saturated fatty acid the salt crystallized either immediately or on cooling in ice water. The salt was readily soluble in warm alcohol and on cooling it separated in small colorless plates. The melting point depends largely upon the rate of heating. When

<sup>2</sup> Personal communication from Prof. T. B. Johnson.

heated slowly in a capillary tube the substance melted at 143–144°C. (uncorrected).

For analysis the substance was dried at 56°C. in a vacuum over phosphorus pentoxide but there was no loss in weight.

0.1280 gm. substance required, Kjeldahl, 5.21 cc. 0.1 N HCl.

0.1162 " " " " 4.80 " 0.1 " "

Found. N 5.70, 5.78 per cent.

Calculated for  $C_{28}H_{40}O_2 \cdot C_6H_{10}SN_2$  (478). N 5.85 per cent.

The percentage of nitrogen in this salt confirms the high molecular weight of the acid.

*Fractionation of the Benzyl Pseudothiourea Salt and Regeneration of the Free Fatty Acid.*—A further quantity of the salt was prepared from 2.8 gm. of the acid. The substance was twice recrystallized from alcohol. The mother liquors were concentrated to about one-half their volume *in vacuo* when another crop of crystals separated, which were filtered off, washed with cold alcohol, and dried.

The substance contained in the last mother liquor was examined as will be described later.

The first crystals weighed 1.7 gm. and melted at 144°C. The second lot weighed 0.8 gm. and melted at 143–144°C. These two fractions were therefore united, weight 2.5 gm., dissolved in 30 cc. of warm alcohol, acidified with hydrochloric acid, diluted with water, and extracted with ether. The ethereal solution was washed with water, filtered, and the ether was distilled. The residue was dried in a vacuum desiccator over sulfuric acid. It formed a faintly yellow oil which weighed 1.7 gm. This fraction was optically inactive since 1.2920 gm. in 15 cc. of alcohol showed no rotation when examined in a 2 dm. tube. The acid was titrated with alcoholic potassium hydroxide with phenolphthalein as indicator.

0.2241 gm. substance required 7.14 cc. 0.1 N KOH.

Found. Mol. wt. 313.

The substance possessed a strong odor of benzyl mercaptan which could not be removed.

The more soluble portion of the salt which was contained in the last mother liquors was decomposed with dilute hydrochloric acid and the fatty acid was recovered in the same manner as just de-



scribed. It was obtained as a light yellow oil that weighed 1.1 gm. The recovery of the total acid was therefore quantitative. The optical rotation was determined by dissolving the total substance, 1.1 gm., in alcohol and the solution was made up to 15 cc. In a 2 dm. tube  $\alpha = + 0.52^\circ$ ;  $[\alpha]_D^{20} = + 3.54^\circ$ . The acid was recovered by evaporating the solvent. This fraction also possessed a strong odor of benzyl mercaptan and in addition it contained some phenolphthalein that had been extracted by the ether from the acidified solution along with the fatty acid. On titration with alcoholic potassium hydroxide the following value was obtained.

0.2643 gm. acid required 7.72 cc. 0.1 N KOH.

Found. Mol. wt. 342.

The higher value found for the molecular weight in this sample was probably due to the admixed impurities as indicated above.

The results of this attempt at fractionation appear to be significant in showing that the acid could be separated into two portions one of which was optically inactive and the other had a greatly increased optical activity. Unfortunately, however, both fractions were contaminated with benzyl mercaptan and it was found to be impossible to remove this impurity.

As a result of this study and the actual isolation of the different cleavage products the composition of the phosphatide may be calculated, 100 parts of phosphatide yielding the following constituents.

	Parts.
Palmitic acid.....	30.5
Oleic acid after reduction to stearic acid.....	12.8
Liquid saturated fatty acid.....	20.9
Glucose.....	13.9
Sugar acid.....	13.8
Glycerophosphoric acid.....	5.4

In the separation of these compounds losses were inevitable, hence the total value is low. The heaviest losses naturally occurred during the separation of the fatty acids.

The nature of the liquid saturated fatty acid and the sugar acid is still unknown. Further work will be carried on, however, in the hope of identifying these compounds.

The author acknowledges with pleasure his indebtedness to Professor T. B. Johnson whose interest in the subject under investigation and whose cooperation made this work possible.

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## THE EFFECTS OF RESPIRATORY GASES UPON THE DENSITY OF BLOOD AND OTHER FLUIDS.\*

By WILLIAM F. HAMILTON AND HENRY G. BARBOUR.

*(From the Department of Physiology and Pharmacology, School of Medicine, University of Louisville, Louisville.)*

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The density changes caused by introducing oxygen or carbon dioxide into blood or, for that matter, any other liquid, do not appear to have been described. The results of an experimental inquiry into these matters have led to findings significant for the physical chemistry of the blood proteins, especially hemoglobin.

Blood and other solutions were equilibrated with various mixtures of carbon dioxide, nitrogen, and oxygen. Differential evaporation was excluded by duplicate solids determinations. Specific gravity differences were determined by the falling drop method (1) (checked under the experimental conditions by a careful pycnometric determination,  $\otimes$  Fig. 1). Blood gas analyses were done by the manometric method of Van Slyke and Neill (2).

Our results are all treated in a single chart (Fig. 1) which shows the effects of various percentages of carbon dioxide upon water ( $\Delta$ ), alkaline salt solution (NaCl 0.7 per cent,  $\text{NaHCO}_3$  0.2 per cent) ( $\square$ ), serum ( $\odot$ ), and whole blood (human  $\bullet$ , dog  $\times$ ), each of which is differently affected by the addition of  $\text{CO}_2$ .

Furthermore, when the  $\text{CO}_2$  is concentrated enough to displace appreciable percentages of  $\text{O}_2$ , the gain in density produced by the  $\text{CO}_2$  is reduced in proportion to the  $\text{O}_2$  loss. The change in density due to variation in  $\text{O}_2$  percentage was estimated from seven different determinations, and fell within the combined error of the two methods. We assumed a linear relation of  $\text{CO}_2$  to density differ-

\* The effects of  $\text{CO}_2$  upon blood density were demonstrated before the Federation of American Societies for Experimental Biology, at Rochester, New York, April, 1927.

ences. The average falling off in density of bloods which had lost oxygen was thus calculated to be  $25 \times 10^{-6}$  for each per cent  $O_2$  difference. A check on this calculation was afforded by the experimental removal *in vacuo* of 8 volumes per cent of oxygen from a saturated (22.5 per cent) sample of dog blood. (The  $CO_2$  change

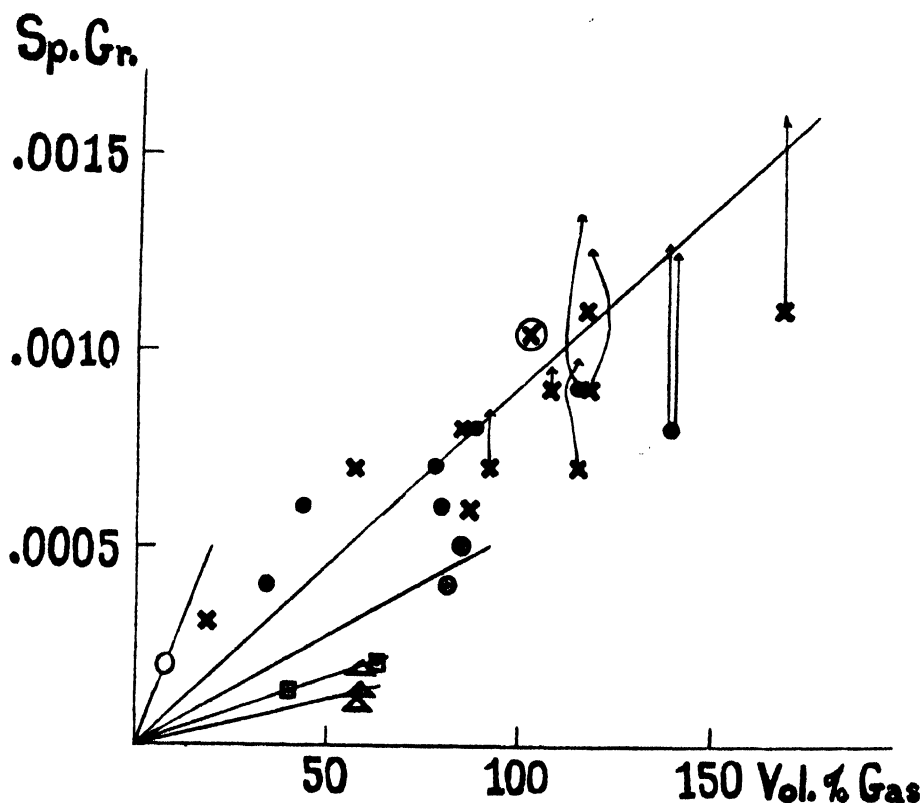


FIG. 1. Effects of carbon dioxide and oxygen upon the density of various fluids. Ordinates, specific gravity change; abscissæ, gas content change, volumes per cent. Effects of carbon dioxide: ● human blood; × dog blood; ⊗ pycnometric determination of dog blood; ○ dog serum; □ alkaline salt solution; △  $H_2O$ ; ○ effect of oxygen upon dog blood. Arrows indicate corrections for lost oxygen.

here was insignificant.) The density difference thus found by direct determination was 0.0002 (○) or  $8 \times 25 \times 10^{-6}$ . The steepest line in the chart represents the effect of  $O_2$  on blood density.

The lines show the following density differences per 1 per cent

of gas content; the last column gives the calculated volume changes.

Solution.	Gas.	Density difference for each volume per cent.	Per cent change in volume of solution for each volume per cent of gas.
H <sub>2</sub> O.....	CO <sub>2</sub>	$2 \times 10^{-6}$	+0.0018
Alkaline salt.....	"	$3 \times 10^{-6}$	+0.0017
Serum.....	"	$5 \times 10^{-6}$	+0.0015
Whole blood.....	"	$9 \times 10^{-6}$	+0.0011
" " .....	O <sub>2</sub>	$25 \times 10^{-6}$	-0.0011

That CO<sub>2</sub> exerts a different effect upon the density of the various solutions, is best explained on the assumption that when the gas goes into these liquids, it changes their respective volumes to a different extent. Thus for each cc. of CO<sub>2</sub> entering 100 cc. of water, the calculated volume increase is 0.0018 cc., but when the same quantity of the gas enters 100 cc. of the alkaline salt solution it increases the volume only 0.0017 cc. Electrostriction consequent upon ionization may explain the difference.

Now in 100 cc. of serum, each cc. of CO<sub>2</sub> increases the volume by a still smaller amount, 0.0015 cc. The added factors in this case include the effect of H ion increase upon the protein molecule and the formation of bicarbonate from the alkali released by the buffers. Thus the formation of acid-protein seems to result in more compact molecular arrangements. Whole blood exhibits a still smaller increase in volume on the addition of CO<sub>2</sub> (0.0011 cc. per 100 cc.). Hemoglobin must in some way be responsible, yet bloods varying in O<sub>2</sub> capacity from 15 to 25 per cent or more, did not show *inter se* any significant variation in density change.

Actual instead of relative shrinkage occurs when we come to the gas which combines chemically with hemoglobin, 100 cc. of blood losing 0.0011 per cent of its original volume for each cc. of added O<sub>2</sub>. Hence oxyhemoglobin may be said to have a more compact molecule than reduced hemoglobin.

#### SUMMARY.

A given amount of CO<sub>2</sub> increases the density of certain fluids in this order:

Water < alkali-salt solution < serum < blood.

Oxygen causes a still greater increase in the density of blood.  
The physical chemistry of these findings is discussed.

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# THE PLASMA PROTEINS OF NORMAL DOGS.

BY C. W. MATTHEW.

(*From the Surgical Service, Henry Ford Hospital, Detroit.*)

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During a recent investigation in which it was desirable to know the content of the various plasma protein fractions of dog plasma the fact became evident that few normal figures were available in the existing literature. It was deemed advisable, therefore, to determine the concentration of the protein fractions in the normal dog.

## *Method.*

The colorimetric method of Wu (1) was used throughout and the specimens were analyzed immediately after taking. All determinations were done in duplicate and only those yielding good checks were accepted.

The animals were carefully examined for signs of infection and all those showing such symptoms were rejected. They were allowed to rest quietly for at least 30 minutes before the withdrawal of blood. An adequate sample of blood may be drawn by cardiac puncture with less discomfort to the animal than from the leg. For this reason venous blood was drawn from the heart, thereby eliminating stasis, which has been shown to increase the concentration of the proteins in the plasma (2, 3). When struggling occurred before or during the procedure the specimen was discarded. These precautions were taken because it has been shown by Rowe (4) that slight muscular activity appreciably increases the plasma protein concentration.

It is known that food has little effect on the plasma proteins (4, 5) but in order to have uniformity all blood samples were taken after a 12 to 14 hour fast. Water was allowed *ad libitum*. Generally 15 cc. of blood were collected in centrifuge tubes over powdered potassium oxalate. The whole blood was then centri-



fused for 20 minutes at 2600 revolutions per minute and the plasma volume determined.

TABLE I.  
*Plasma Proteins of Normal Dogs.*

Experiment No.	Plasma volume.	Fibrin.	Albumin.	Globulin.	Total protein.	Experiment No.	Plasma volume.	Fibrin.	Albumin.	Globulin.	Total protein.
	per cent	per cent	per cent	per cent	per cent		per cent	per cent	per cent	per cent	per cent
1	64.3	0.64	5.31	2.04	7.99	26	46.0	0.35	4.64	2.19	7.18
2	70.6	0.68	4.78	2.54	8.00	27	57.5	0.56	4.43	1.89	6.88
3	53.3	0.47	5.35	2.14	7.96	28	43.4	0.45	4.45	2.90	7.80
4	59.5	0.41	5.18	2.37	7.96	29	66.3	0.36	5.77	2.24	8.17
5	60.9	0.45	4.13	2.32	6.90	30	59.0	0.45	4.92	2.09	7.46
6	62.6	0.47	4.23	2.32	7.02	31	62.7	0.57	4.61	1.98	7.16
7	64.3	0.50	4.80	2.00	7.30	32	58.1	0.33	4.85	2.18	7.36
8	61.3	0.45	4.62	2.10	7.17	33	67.1	0.35	4.58	2.52	7.45
9	56.6	0.42	5.10	2.00	7.52	34	74.4	0.42	4.81	1.87	7.10
10	60.0	0.38	4.20	1.98	6.56	35	64.9	0.45	5.02	1.94	7.41
11	60.0	0.41	5.00	1.97	7.38	36	70.4	0.47	4.58	2.16	7.21
12	64.4	0.42	4.30	2.00	6.72	37	63.7	0.46	4.30	2.10	6.86
13	56.7	0.47	5.00	1.98	7.45	38	42.1	0.46	5.10	2.12	7.68
14	59.6	0.38	4.60	2.00	6.98	39	54.3	0.48	4.30	2.10	6.88
15	63.0	0.45	4.52	2.00	6.97	40	58.4	0.50	4.40	1.78	6.68
16	60.1	0.33	4.62	1.86	6.81	41	60.1	0.47	4.30	2.36	7.13
17	59.1	0.46	4.70	1.80	6.69	42	64.0	0.49	4.29	2.22	7.00
18	49.6	0.46	4.58	2.10	7.14	43	57.5	0.42	4.42	2.11	6.95
19	49.6	0.36	4.62	2.14	7.12	44	59.0	0.42	4.64	2.10	7.16
20	54.0	0.39	4.20	2.00	6.59	45	62.3	0.51	4.31	2.36	7.18
21	59.9	0.38	4.62	1.86	6.86	46	64.2	0.38	4.52	2.12	7.02
22	61.3	0.46	5.00	1.96	7.42	47	52.8	0.42	4.68	2.00	7.10
23	65.9	0.42	4.81	1.91	7.14	48	60.0	0.60	4.20	2.49	7.29
24	66.9	0.47	5.07	2.24	7.78	49	58.6	0.41	4.51	2.36	7.29
25	68.0	0.42	4.58	1.89	6.89	50	59.5	0.36	4.44	2.42	7.22
Average.....	60.	0.45	4.64	2.12	7.23						

To determine the constancy of the protein concentration in individual animals, several dogs were kept under observation from 4 to 72 hours.

*Results.*

The results of analysis of blood from 50 animals are given in Table I. It may be seen that the fibrin values average 0.45 per cent and range from 0.33 to 0.68 per cent. The albumin content yields an average of 4.64 per cent and varies from 4.13 to 5.57 per cent. The globulin values average 2.12 per cent, the extremes being 1.78 and 2.90 per cent. The total protein (obtained by

TABLE II.  
*Constancy of Plasma Proteins.*

Experiment No.	Time.	Fibrin.	Albumin.	Globulin.	Total protein.
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
8		0.45	4.62	2.10	7.17
	4	0.43	4.61	2.08	7.12
19		0.36	4.62	2.14	7.12
	8	0.38	4.61	2.12	7.11
	24	0.38	4.59	2.16	7.13
24		0.47	5.07	2.24	7.78
	4	0.47	5.10	2.12	7.69
	8	0.48	5.04	2.20	7.72
	24	0.47	5.01	2.19	7.67
36		0.47	4.58	2.16	7.21
	8	0.45	4.50	2.20	7.15
	24	0.45	4.57	2.18	7.20
	48	0.49	4.49	2.22	7.20
43		0.42	4.42	2.11	6.95
	24	0.43	4.30	2.24	6.97
	48	0.40	4.40	2.16	6.96
	72	0.40	4.50	2.05	6.95

adding the results of the above three determinations) average 7.23 per cent and vary from 6.56 to 8.17 per cent. The average plasma volume is 60.0 per cent and varies from 42.1 and 74.4 per cent.

Table II shows the constancy of the protein concentration over a period of hours and days. It may be seen that the variations are well within the limits of experimental error.

## SUMMARY.

The concentration of the plasma proteins was determined in 50 normal dogs.

It was found that the proteins remain constant in any one animal over a period of several days.

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## CONDENSATION PRODUCTS OF ACETOACETIC ESTER.

### III. A SERIES OF NEW COMPOUNDS OF GLUCOSE. FURTHER STUDIES IN ANTIKETOGENESIS.

By EDWARD S. WEST.

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)

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Shaffer and others (1) have suggested that glucose may be anti-ketogenic in human metabolism because it, or a derivative, combines with acetoacetic acid or other ketogenic molecule to form a substance more easily oxidized than the unchanged acetone bodies. Shaffer and Friedemann (2) have reported various *in vitro* analogies which may be interpreted according to this idea.

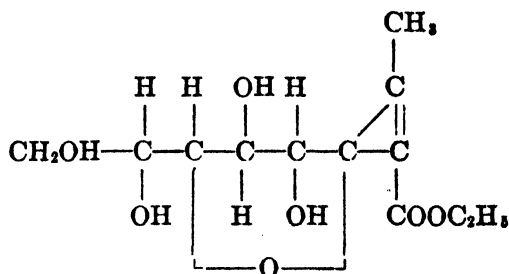
One method of testing the possible validity of this hypothesis as to the mechanism of antiketogenesis is to prepare condensation products of acetoacetic acid with sugars or their derivatives and to ascertain their properties, particularly their relative ease of oxidation *in vitro* and *in vivo*. This the writer has undertaken to do. Two reports have already been published on condensation products of glyoxal and acetoacetic ester (3) and on the *in vitro* oxidation of condensation products of acetoacetic ester in general (4). These compounds previously reported and those described below exhibit interesting properties apart from their possible bearing upon the problem of antiketogenesis.

The present communication deals with the preparation and some of the properties of a series of condensation products of glucose and acetoacetic acid and its ethyl ester together with a consideration of their possible relationship to antiketogenesis. A general theory as to the mechanism of certain types of catalysis and of enzyme action is also presented from the electronic view-point.

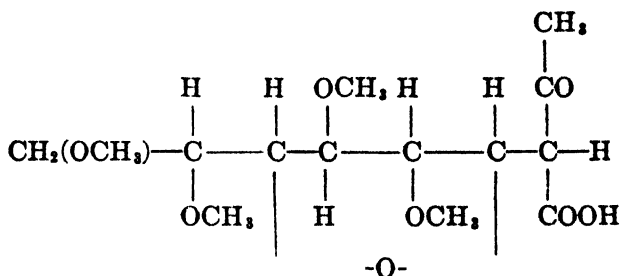
Biginelli (5) observed the formation of a compound,  $C_{16}H_{20}O_8$ , m.p. 189–190°, as a result of the action of acetoacetic ester and alcoholic ammonia upon glucose. Apparently he did not suc-

ceed in determining the constitution of the compound. Schiff prepared a number of compounds of sugars with aldehydes and ketones, including one of glucose and acetoacetic ester (6). These compounds Schiff found to be not condensation but addition products easily broken up into their constituents. Condensation products of sugars involving carbon to oxygen linkage are numerous, but condensation with the production of carbon to carbon linkage has rarely been effected. It is believed that the series of condensation products of glucose and acetoacetic ester and acid described below is representative of the latter type of compounds.

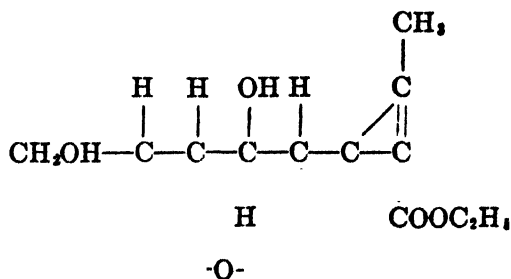
The fundamental compounds here described and their tentative structures are the following.



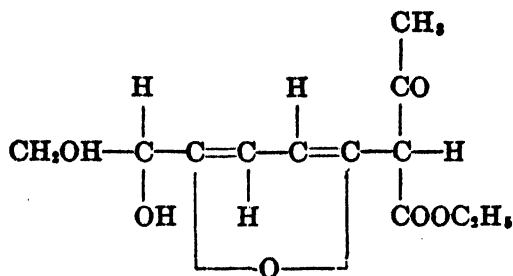
I. Glucose cycloacetoacetic ester (and acid).



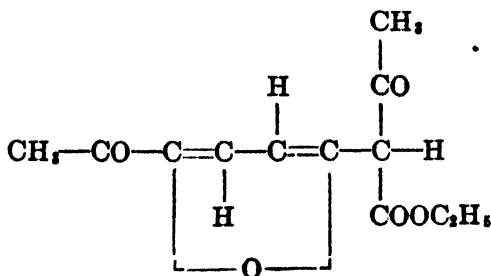
II. Tetramethylglucose acetoacetic acid.



III. Anhydroglucose cycloacetoacetic ester (and acid).



IV. Dianhydroglucose acetoacetic ester.



V. Trianhydroglucose acetoacetic ester.

As pointed out later there are certain details of configuration which have not been established.

#### A. Glucose Cycloacetoacetic Ester and Acid.

Glucose condenses with acetoacetic ester in the presence of zinc chloride to form a beautifully crystalline compound,  $\text{C}_{12}\text{H}_{18}\text{O}_7$ , corresponding to the interaction of 1 molecule each of glucose and acetoacetic ester with the loss of 2 molecules of water. The compound contains one ethoxyl group. When saponified it yields a crystalline acid,  $\text{C}_{10}\text{H}_{14}\text{O}_7$ , of neutral equivalent 246. The ester and acid do not reduce alkaline copper solutions nor do they give reactions characteristic of the carbonyl group. These facts indicate that the aldehyde group (or glucosidic hydroxyl) of glucose is concerned in the condensation and also that the carbonyl group of the acetoacetic ester chain has disappeared. Prolonged boiling of the ester with 10 per cent sodium hydroxide does not cause the elimination of carbon dioxide. Upon acidification the corresponding acid is obtained. Both ester and acid decolorize aqueous permanganate quickly in the cold suggesting the presence of an unsaturated linkage. That the compounds contain four hydroxyl groups has been established by the prep-

aration of tetraacetyl derivatives and a tetramethyl derivative of the acid.

When boiled with dilute hydrochloric acid the compounds become powerfully reducing toward alkaline copper reagents, dyestuffs, etc., and give reactions with sodium nitroprusside, phenylhydrazine, and alkaline iodine solution characteristic of the  $\text{CH}_3\text{CO}$  group. If the acid solution be made weakly alkaline and heated it loses its reducing powers and gives none of the reactions of the  $\text{CH}_3\text{CO}$  group. Upon reacidification and short boiling, however, all of these properties return. As will be shown in the experimental part the reducing compound formed here is probably anhydroglucose acetoacetic ester. These facts seem to indicate the presence of an unstable carbon ring involving the acetoacetic acid chain. Hydrolysis with acid brings about rupture of the ring and the appearance of the  $\text{CH}_3\text{CO}$  group, while treatment with alkali causes the reverse. A derivative of the ester containing the normal acetoacetic acid chain but modified glucose molecule has been prepared by the action of concentrated hydrochloric acid at room temperature (4). This compound is described in detail later in the discussion. The reaction of glucose and acetoacetic ester in the presence of zinc chloride then probably forms glucose cycloacetoacetic ester (I).<sup>1</sup>

It is uncertain whether the compound is a derivative of  $\alpha$ - or  $\beta$ -glucose. According to these reactions the aldehydic hydroxyl of the  $\alpha$  or  $\beta$  form of glucose condenses with an active methylene hydrogen atom of acetoacetic ester with the elimination of water and formation of glucose acetoacetic ester. The hydroxyl of the enolic form of this compound then condenses with hydrogen of the glucose chain to form glucose cycloacetoacetic ester (I), with an unstable three- or four-membered carbon ring containing one double bond. Compounds containing such rings have been studied by Feist (7) and Willstätter and Bruce (8). Perkin and Simonsen (9) have pointed out that the nature of the substituent groups in cyclopropane and cyclobutane rings largely determines their stability. The ring present in glucose cycloacetoacetic ester and acid is so readily ruptured by acid hydrolysis

<sup>1</sup> This configuration differs somewhat from that tentatively proposed in a previous publication (4).

that the writer is inclined to favor the presence of the three-membered ring. The instability of the ring in tetramethyl glucose cycloacetoacetic acid is such that it is hydrolyzed by its own acidity when boiled in aqueous solution. Ordinarily cyclopropane and cyclobutane rings are not readily oxidized by permanganate but the cyclopropene and cyclobutene rings are (7, 8). These facts are in accord with the properties of glucose cycloacetoacetic ester and the formula suggested for it (I). The ester possesses an  $[\alpha]_D$  of  $-19^\circ$  and the acid  $-21.5^\circ$  in methyl alcohol. If the compounds are derivatives of  $\alpha$ -glucose a very marked change in rotation has been effected by substitution of the acetoacetic acid chain for the aldehydic hydroxyl group of glucose and subsequent ring formation. Ring formation is known to have a pronounced effect upon rotation when it involves an asymmetric carbon atom (10). Experiments to test more decisively the nature of the ring in these derivatives are in progress.

#### *B. Tetramethylglucose Cycloacetoacetic Acid.*

The main interest attached to this compound is in connection with the ease of opening the carbon ring involving the acetoacetic acid chain and the unusual reducing power of the substance formed. The compound itself does not reduce alkaline copper solutions but does reduce aqueous permanganate instantly in the cold. It gives none of the reactions of the carbonyl group. When an aqueous solution is refluxed for several hours, however, the carbon ring is opened as shown by the observations that the solution gives a copious and instantaneous precipitate of iodoform with alkaline iodine, a characteristic carbonyl reaction with nitroprusside, and an immediate precipitate with phenylhydrazine. At the same time the solution acquires the power of *reducing strongly alkaline copper solutions quickly in the cold*. A carbonate copper solution, as Benedict's, is reduced in the cold by the acid in several minutes. The reducing compound has not as yet been isolated in pure condition though attempts are being made to accomplish this. The evidence seems to indicate that it is the simple hydrolytic product of tetramethylglucose cycloacetoacetic acid in which the carbon ring has been opened; namely, tetramethylglucose acetoacetic acid (II).



*C. Anhydroglucose Cycloacetoacetic Ester and Acid.*

When glucose cycloacetoacetic ester (I) is treated with concentrated hydrochloric acid at 0°C. for a few minutes and the reaction product separated as described in the experimental discussion a golden yellow syrup is obtained of the composition  $C_{12}H_{16}O_6$ . Upon saponification the ester yields an acid  $C_{10}H_{12}O_6$ , neutral equivalent 228. The ester and acid correspond to the loss of a molecule of water from glucose cycloacetoacetic ester and acid. The compounds contain two hydroxyl groups as shown by acetylation. Boiling alkali does not eliminate carbon dioxide.

The compounds are non-reducing toward alkaline copper solutions but reduce aqueous permanganate in the cold. Boiled with dilute hydrochloric acid they become reducing to copper solutions and show reactions of the  $CH_3CO$  group just as do the parent substances glucose cycloacetoacetic ester and acid. The conclusion seems justified that the only change induced in the molecule of glucose cycloacetoacetic ester by treatment with concentrated hydrochloric acid at 0°C. is the loss of a molecule of water from two hydroxyl groups of the glucose chain and the formation of anhydroglucose cycloacetoacetic ester (III).

The  $[\alpha]_D$  values for anhydroglucose cycloacetoacetic ester and acid are  $-89.9^\circ$  and  $-111.7^\circ$  as compared with  $-19^\circ$  and  $-21.5^\circ$  for glucose cycloacetoacetic ester and acid. This represents a very great change in optical rotation and is fully in accord with ring formation involving an asymmetric carbon atom (10). The determination of the specific hydroxyl groups concerned in anhydride formation must await future investigation.

Anhydroglucose cycloacetoacetic acid is also formed when glucose cycloacetoacetic acid is boiled in aqueous solution, preferably in the presence of a trace of hydrogen chloride. The acetoacetic acid chain and cyclopropene ring evidently exert a marked influence upon certain hydroxyl groups of the glucose chain in favoring anhydride formation.

*D. Dianhydroglucose Acetoacetic Ester.*

If glucose cycloacetoacetic ester (I) be treated with concentrated hydrochloric acid at room temperature for some time

instead of at  $0^{\circ}\text{C}$ . and the reaction product separated, a yellow syrup,  $\text{C}_{12}\text{H}_{16}\text{O}_6$ , is obtained, isomeric with anhydroglucose cycloacetoacetic ester (III), but possessing powerful reducing properties. The substance reduces Fehling's solution *quickly in the cold*, and gives characteristic reactions for the  $\text{CH}_3\text{CO}$  group. It is decomposed and resinified by alkalis even in the cold, and by dilute acids upon heating. The corresponding acid has not been obtained. The hydrazine derivatives are unstable and soon resinify. Analysis shows that the substance contains one ethoxyl and two hydroxyl groups. Since the compound reacts with phenylhydrazine, gives the characteristic blue-green coloration with nitroprusside, and yields iodoform when treated with iodine and alkali the conclusion seems justified that the ring involving the acetoacetic acid chain has been opened. This would involve the addition of a molecule of water to the original compound,  $\text{C}_{12}\text{H}_{18}\text{O}_7$ , with the formation of  $\text{C}_{12}\text{H}_{20}\text{O}_8$ . In order to obtain the compound  $\text{C}_{12}\text{H}_{16}\text{O}_6$  from the latter the loss of 2 molecules of water from the glucose chain would be necessary. Considering the fact that the glucose chain still contains two hydroxyl groups the loss of water must be accomplished by a process of unsaturation and not anhydride formation as in case of its isomer, anhydroglucose cycloacetoacetic ester. The properties of the compound indicate unsaturation. The isomer, anhydroglucose cycloacetoacetic ester, appears to be an intermediate in the formation of the compound. If the action of hydrochloric acid is prematurely interrupted more or less of the isomer is obtained. Also dianhydroglucose acetoacetic ester may be prepared by treatment of the latter with concentrated hydrochloric acid at room temperature.

Apparently the action of hydrochloric acid at room temperature upon glucose cycloacetoacetic ester (I) first causes the formation of anhydroglucose cycloacetoacetic ester (III), followed by hydrolysis of the propene ring with the formation of anhydroglucose acetoacetic ester.<sup>2</sup> It may well be that the anhydride ring is stabilized by the propene ring and is hydrolyzed immediately upon the destruction of it. The next step then consists in

\* Evidence for the existence of this substance is presented in the experimental part.

the hydrolysis of the anhydride ring followed by the loss of 2 molecules of water from the glucose chain with the production of two double bonds and the formation of dianhydroglucose acetoacetic ester (IV).

The exact positions of the double bonds must await further investigation. The relation of trianhydroglucose acetoacetic ester to dianhydroglucose acetoacetic ester, as pointed out later, suggests that the double bonds are correctly placed. It has been assumed that the loss of water takes place in the hydrofurfurane ring since unsaturation greatly increases the stability of the ring (11). The compound contains 1 asymmetric carbon atom<sup>3</sup> in the glucose chain in contrast to 5 for glucose cycloacetoacetic ester. The presence of double bonds adjacent to an asymmetric carbon atom is known to increase optical rotation and it is not surprising that the substance shows a higher rotation than glucose cycloacetoacetic ester. Neither is it unexpected that the rotation of the compound is considerably less than that of anhydroglucose cycloacetoacetic ester with 5 asymmetric carbon atoms of which at least 1, or perhaps 2, are involved in ring formation.

#### *E. Trianhydroglucose Acetoacetic Ester.*

During the distillation of dianhydroglucose acetoacetic ester (IV) some of it loses a molecule of water and passes into a yellow crystalline substance of the composition  $C_{12}H_{14}O_6$ . Analysis shows the presence of one ethoxyl and one hydroxyl group. The compound gives the characteristic reactions of the  $CH_3CO$  group and is easily soluble in dilute alkalis which points to the absence of a ring involving the acetoacetic acid chain. It is strikingly distinguished from dianhydroglucose acetoacetic ester in that it is not resinified by boiling with dilute alkali or acid.

Assuming the structure of dianhydroglucose acetoacetic ester to be as postulated above (IV), the formula of trianhydroglucose acetoacetic ester formed from it by the loss of a molecule of water may be represented by (V).

<sup>3</sup> There is the possibility of two optical isomers as the result of the appearance of an asymmetric carbon atom in the acetoacetic acid chain. If present they probably represent a racemic mixture since trianhydroglucose acetoacetic ester, which presumably contains no other asymmetric carbon atom than the one in the acetoacetic acid chain, is optically inactive.

Since the compound does not reduce Tollens' reagent it presumably does not contain an aldehyde group. This suggests that the loss of water from dianhydroglucose acetoacetic ester involves the hydroxyl of the terminal carbon of the glucose chain.

The hydroxyl group determined in the compound forms a very easily hydrolyzed acetyl derivative. It very likely is the enolic hydroxyl of the acetoacetic acid chain.

The observation that dianhydroglucose acetoacetic ester is optically active while trianhydroglucose acetoacetic ester is *optically inactive* is evidence in support of the above formulation. If dianhydroglucose acetoacetic ester contains only 1 asymmetric carbon atom in the glucose chain, as postulated, and this carbon atom becomes involved in double bond formation, the optical activity should disappear. This accords with the observation. The formation of trianhydroglucose acetoacetic ester from dianhydroglucose acetoacetic ester by the loss of water cannot involve ring formation because this would not destroy the asymmetric carbon atom but would probably increase the optical rotation. It is difficult to picture the loss of 3 molecules of water from the glucose chain of glucose acetoacetic ester with the formation of three double bonds in any way other than that represented. The number of hydroxyl groups present in the dianhydro and trianhydro derivatives as compared with the molecules of water lost in the glucose chain can be accounted for only by assuming the formation of double bonds. Lastly, the phenomena of optical rotation referred to above, as far as the writer is able to see, can be explained on the basis of no other formulation.

It will be observed in the experimental discussion that the analyses of the phenylhydrazine derivative of trianhydroglucose acetoacetic ester do not agree with the calculated values for either the mono- or dihydrazone, nor the monohydrazone minus either water or alcohol. They do agree quite well however for the monophenylhydrazone minus the equivalent of CO. This is a rather unexpected observation. Should CO be split from carbon atom 5 of the glucose chain and the methyl group become attached to the furane ring in its place the analytical figures would be satisfied, but this is a rather unreasonable guess, and we must await future work to explain the discrepancy. That

phenylhydrazine reacted with the CO group of the acetoacetic acid chain and not a ketone group of the glucose chain is shown by the observation that the hydrazone is insoluble in alkali. The compound would be expected to form a dihydrazone, but under the conditions used only the modified monohydrazone was produced. It may be that with other conditions a dihydrazone would form, but this point has not yet been tested.

#### DISCUSSION.

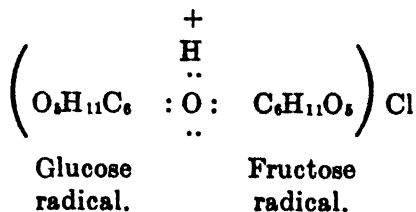
It was pointed out in a previous publication (4) dealing with the theory of oxidation of some condensation products of acetoacetic ester that the ease of oxidation of these compounds is probably due to an increased electronic activity of the methylene carbon atom of the acetoacetic acid chain as a result of replacing hydrogen by the various groups. It was shown at that time that a condensation product of glucose and acetoacetic ester (dianhydroglucose acetoacetic ester) possesses remarkable reducing power. It is not only more easily oxidized *in vitro* than acetoacetic acid but is more easily oxidized than glucose itself. This reducing power was likewise attributed to the "loosening," or activation, of the electrons of the methylene carbon of acetoacetic acid by attachment of the glucose radical to it, causing them to be more readily lost to an oxidizing atom. This concept gathers weight when tetramethylglucose acetoacetic acid is considered. It may reasonably be assumed that no part of the tetramethylglucose radical readily loses electrons or is easily oxidized. This is not necessarily true in case of the dianhydroglucose radical of dianhydroglucose acetoacetic ester previously reported. Also the carbon of the  $\text{CH}_3\text{CO}$  and  $\text{COOH}$  groups of the acetoacetic acid chain would not be expected to show such striking powers of reduction. We may logically conclude therefore that the substitution of a tetramethyl glucose radical for a methylene hydrogen of the acetoacetic acid chain has so decreased the attraction of the carbon atom to which this radical is attached for some of its electron pairs that they are readily lost and the compound easily oxidized. This stepping up of the reduction potential or ease of oxidation of the acetoacetic acid molecule by condensation with glucose or a derivative is fully in accord with Shaffer's theory of the mechanism of antiketogenesis in human metabolism. In this connec-

tion it is of interest to note that the *in vitro* effect of the glucose radical on the ease of oxidation of the acetoacetic acid chain from an electronic view-point concerns precisely the carbon atom which according to Knoop's theory of  $\beta$  oxidation (12) is resistant to oxidation *in vivo*.

Hynd's observation (13) that acetoacetic acid is effective in relieving symptoms induced in mice by glucosone is of interest in this connection. There is the possibility of complex formation in this case with the production of a substance more easily oxidized than one or either component and therefore perhaps less toxic. As pointed out by Hynd glucosone may represent a sugar derivative concerned in normal carbohydrate metabolism and antiketogenesis.

It is plausible to suppose that *various oxidations* may occur in the animal body in which one compound aids the oxidation of another by combination with it *forming a complex more easily oxidized by the body than one or both constituents*. This may reasonably be true of rather "loose" combinations also. In terms of modern ideas of valence this concept simply means that certain valence electron pairs are held less firmly by the atomic nuclei in the complex than they were in the constituent molecules. Kharasch and Marker (14) have shown that substitution of various radicals for hydrogen of methane alters the attraction of the methane carbon for its other pairs of electrons.

It seems to the writer that many catalytic actions besides those of oxidation both *in vitro* and *in vivo* may have some such chemical basis. It may well be, for example, that a loose oxonium compound of sucrose and hydrogen chloride



involving the glucosidic oxygen atom connecting the glucose and fructose portions of the molecule, as shown, represents a combination or complex in which the electron pairs shared by carbon with the glucosidic oxygen atom are much less firmly held

than in the case of unmodified sucrose, thus facilitating hydrolytic cleavage. Some such effect is clearly to be expected from such combinations. In the particular instance cited since the  $\text{Cl}^-$  is supposedly almost completely dissociated from the complex and the  $\text{H}^+$  *very little dissociated* it is probable that the *loosening effect* on the *electron pairs* is *largely due* to the  $\text{H}^+$ . The amount of  $\text{H}^+$  combined in oxonium linkage would be directly *proportional to the hydrogen ion concentration*, which is *exactly in accord* with the *catalytic effect* of acids in such cases. *An oxidative or hydrolytic enzyme in the animal body* might possibly function similarly by uniting with the substrate and loosening the attraction of certain atomic nuclei for electron pairs in such a way that the desired change is accomplished. A possible mechanism for the specific dynamic effect of proteins or amino acids in metabolism would be complex formation with other metabolites thereby causing certain electron pairs to be held less firmly and the metabolites to be more easily and rapidly oxidized than when uncombined.

The activation of molecular oxygen by peroxide formation which is supposedly of physiological importance is very likely the converse of the above mechanism. Here *the attraction of oxygen for electrons is increased* by combination with carbon atoms with the result that it takes up electrons more readily and is a more powerful oxidizing agent than molecular oxygen.

We are very much interested in the metabolism of the compounds reported above and hope to publish the results of our studies in the near future.

It is likely that by the use of proper procedures, various transformations of these compounds of interest in the sugar group can be effected. No such studies have been undertaken.

Systematic attempts to extend the reaction to other sugars have not been made, though if it were characteristic of glucose alone it would be a remarkable thing. Other 1, 3-diketones than acetoacetic ester and possibly many compounds which contain mobile hydrogen united to carbon may condense similarly with glucose and other sugars. It is hoped that other workers will investigate such possibilities.

## EXPERIMENTAL.

*Materials.*—Acetoacetic ester, practical grade supplied by Eastman Kodak Company. Glucose, a commercial grade known as cerelese.

*Glucose Cycloacetoacetic Ester.*

*Preparation.*—Glucose (100 gm.) and fused zinc chloride (50 gm.), finely powdered and mixed, are placed in a short neck Kjeldahl flask (300 cc.) with acetoacetic ester (50 gm.) and 95 per cent ethyl alcohol (50 cc.).

The mixture is heated in a boiling water bath with violent stirring until practically all solid disappears and a heavy yellow syrup results (approximately 15 minutes). The syrup is poured into 400 cc. of water and placed in the cold room. Very soon a bulky mass of glistening needle-shaped crystals separates. With slow crystallization beautiful rosettes form. After standing several hours in the cold the crystals are separated as thoroughly as possible from the mother liquor by suction and recrystallized three times from hot water (a small amount of norit being used). There is little loss in recrystallization from moderate amounts of water. Yield about 20 gm. The yield is improved by pouring the syrup into mother liquor from the recrystallization of a previous preparation. The above procedure is the result of many experiments in which proportion of reactants, temperature, and time of heating were varied. The presence of alcohol was found practically to double the yield. Supposedly it minimizes decomposition of acetoacetic ester or the condensation product or both.

The compound is difficultly soluble in cold water and readily in hot, slightly soluble in ether and chloroform, and soluble in alcohol, ethyl acetate, and pyridine.

*Analysis.* 0.3259, 0.3313 gm. substance:  $\text{CO}_2$  0.6266, 0.6386,  $\text{H}_2\text{O}$  0.1894, 0.1969.

Calculated for  $\text{C}_{12}\text{H}_{18}\text{O}_7$ . C 52.53, H 6.61.

Found.

" 52.29, 52.42 H 6.45, 6.59.

Ethoxyl (Zeisel). 0.2411, 0.2813 gm. substance: AgI 0.2005, 0.2342.

Calculated for one ethoxyl group, 16.41; found, 15.94, 15.96.

*Optical Rotation.*—Solvent  $\text{CH}_3\text{OH}$ . 1.50 gm. of compound in 100 cc. Tube 2 dm.  $\alpha$ ,  $-0.57^\circ$ .



$$[\alpha]_D^{20} = \frac{-0.57 \times 100}{2 \times 1.5} = -19.00^\circ.$$

The compound showed no mutarotation. Temperature was controlled by a water-jacketed tube.

*Chemical Properties of Glucose Cycloacetoacetic Ester.*

The compound does not reduce hot alkaline copper solutions (Fehling's, Benedict's, Shaffer-Hartmann) or Tollens' reagent, but readily decolorizes neutral permanganate in the cold. It does not form an oxime or phenylhydrazine derivative, nor does it give iodoform when treated with iodine and alkali. The nitroprusside test for the carbonyl group is negative.

*Action of Dilute Acid and Alkali. Opening and Closure of the Ring Involving the Acetoacetic Acid Chain.*

1. *Action of Acid. Opening of the Ring.*—When the compound is boiled for a short time with dilute hydrochloric acid apparently a linkage is hydrolyzed to form the  $\text{CH}_3\text{CO}$  group. If this acid solution is made alkaline and warmed the process is reversed. Reacidification and warming again cause the reappearance of the  $\text{CH}_3\text{CO}$  group. This may be repeated several times. The following experiment is typical. Approximately 0.7 gm. of compound was gently boiled with 25 cc. of  $\text{N HCl}$  for 15 to 20 minutes and cooled. The solution became very faintly yellow and slightly opaque due to the separation of minute droplets of an oil.

The solution produced an intense reduction of hot alkaline copper reagents when added to them, but the amount of reduction was much less when the solution was mixed with the cold reagent and then heated. Apparently the alkali of the reagent destroyed the reducing power of the compound. When sodium nitroprusside was added to the acid solution and then alkali a fleeting red coloration appeared which changed to a blue-green upon acidification with glacial acetic acid. The addition of  $\text{I}_2$  in  $\text{KI}$  to the acid solution followed by alkali gave a copious precipitate of iodoform (microscopic identification). The phenylhydrazine reaction for the carbonyl group according to Mulliken (15) gave an immediate positive test.

*2. Action of Alkali. Closure of the Ring.*—The acid solution above was made weakly alkaline with dilute NaOH and heated to boiling. When tested as before for the presence of the  $\text{CH}_3\text{CO}$  group all reactions were negative. Also the compound had lost its power of reducing hot alkaline copper solutions. The inactive alkaline solution was acidified with dilute HCl as at first and boiled for a few minutes. After this treatment all reactions for the  $\text{CH}_3\text{CO}$  group were positive, and the solution regained its reducing power.

Attempts to isolate the reducing open ring compound formed by hydrolysis with dilute HCl have been unsuccessful. When the reducing acid solution was neutralized with sodium bicarbonate and the oil extracted with chloroform it was found to have largely lost its activity. The oil was chiefly anhydroglucose cycloacetoacetic ester because when saponified it gave a large amount of anhydroglucose cycloacetoacetic acid described below. The determination of C and H on the oil gave C 56.20, H 6.26. Calculated for  $\text{C}_{12}\text{H}_{16}\text{O}_6$ , C 56.23, H 6.29. The reducing compound present in the hydrolyzed solution was probably anhydroglucose acetoacetic ester which with increasing pH undergoes ring closure with the formation of anhydroglucose cycloacetoacetic ester. Apparently ring closure takes place with great ease at a slightly alkaline pH.

*Elimination of Carbon Dioxide from Glucose Cycloacetoacetic Ester by Acid and Alkali.*

*1. Action of Acid.*—The compound was heated under a reflux with 3 N  $\text{H}_2\text{SO}_4$  for 3 hours in a current of nitrogen gas. The  $\text{CO}_2$  liberated was absorbed in a tower containing 0.5 N NaOH and estimated by addition of  $\text{BaCl}_2$  and titration in the usual way.

0.8545 gm. of the substance liberated 0.106 gm. of  $\text{CO}_2$ . Calculated for one  $\text{CO}_2$  group, 0.1528 gm., per cent of theoretical 69.2. A similar experiment in which 6 N  $\text{H}_2\text{SO}_4$  was used gave 74.4 per cent of the theoretical  $\text{CO}_2$  elimination. The compound undergoes profound resinification and decomposition when heated with moderately strong acid.

*2. Action of Alkali.*—1.00 gm. of the compound was boiled for 18 hours with 10 per cent NaOH under reflux. Analysis as above showed that no  $\text{CO}_2$  had been eliminated. When the alkaline

solution was acidified glucose cycloacetoacetic acid separated. The compound is exceedingly stable toward the action of hot alkali and apparently suffers only saponification.

*Tetraacetylglucose Cycloacetoacetic Ester.*

*Preparation.*—Glucose cycloacetoacetic ester (10 gm.), acetic anhydride (40 cc.), and pyridine (40 cc.), are mixed and heated under a reflux on a boiling water bath for 5 to 6 hours. The pyridine and acetic anhydride are distilled off, the last portions under a vacuum from a water bath at 100°C. The resulting heavy syrup, upon cooling, sets to a crystalline mass. It is dissolved in hot 60 to 70 per cent alcohol, a pinch of norit added, filtered, and allowed to cool. Beautiful prismatic platelets separate which after drying over  $\text{CaCl}_2$  in a vacuum melt at 84°C. Yield about 12 gm.

*Analysis.* 0.2052, 0.2031 gm. substance:  $\text{H}_2\text{O}$  0.1134, 0.1096,  $\text{CO}_2$  0.4082, 0.4040.

Calculated for  $\text{C}_{20}\text{H}_{28}\text{O}_{11}$ . C 54.27, H 5.92.

Found. " 54.24, 54.24, H 6.14, 5.99.

Acetyl. 1.5293 gm. substance, hydrolyzed with 30 per cent  $\text{H}_3\text{PO}_4$ . Acetic acid found, 138.01 cc. 0.1 N. Calculated for four acetyl groups, 138.40 cc. 0.1 N.

*Optical Rotation.*—Solvent  $\text{CHCl}_3$ . Tube 2 dm. 2.0078 gm. substance in 100 cc.  $\alpha$ ,  $-1.48^\circ$ .

$$[\alpha]_D^{20} = \frac{-1.48 \times 100}{2 \times 2.0078} = -36.85^\circ.$$

The compound does not reduce hot alkaline copper reagents.

*Glucose Cycloacetoacetic Acid.*

*Preparation.*—Glucose cycloacetoacetic ester (10 gm.) is treated with 10 per cent  $\text{NaOH}$  (25 cc.) and allowed to stand 15 hours at 37°, or heated 2 hours under a reflux on a boiling water bath. The thoroughly cooled solution is acidified to methyl orange with  $\text{H}_3\text{PO}_4$  whereupon the whole sets to a mass of needle-shaped crystals. The mass is broken up with a little cold water in a mortar, the crystals separated by suction, and recrystallized three times from hot water, a small amount of norit being used, and care being taken to cool (5°C.) the solution thoroughly at each

crystallization. With slow crystallization the acid separates in beautiful rosettes of needles. After drying in a vacuum over  $\text{H}_2\text{SO}_4$  the compound, heated rapidly, melts at  $160\text{--}161^\circ$  with decomposition.

1.3 gm. of acid dissolve in 100 cc. of  $\text{H}_2\text{O}$  at  $21^\circ\text{C}$ . It is slightly soluble in cold alcohol, readily in hot, and difficultly soluble in chloroform and ether.

*Analysis.* 0.2843, 0.2744 gm. substance:  $\text{CO}_2$  0.5076, 0.4902,  $\text{H}_2\text{O}$  0.1473, 0.1412.

Calculated for  $\text{C}_{10}\text{H}_{14}\text{O}_7$ . C 48.75, H 5.73.

Found. " 48.56, 48.58, H 5.75, 5.71.

0.2046 gm. substance: 8.52 cc. 0.1 N NaOH. Neutral equivalent 242.48; calculated 246.11.

#### *A. Optical Rotation of the Acid.*

1. Solvent  $\text{CH}_3\text{OH}$ . 1.346 gm. substance in 100 cc.  $\alpha$ ,  $-0.58^\circ$ .

$$[\alpha]_D^{25} = \frac{-0.58 \times 100}{2 \times 1.346} = -21.54^\circ.$$

2. Solvent  $\text{H}_2\text{O}$ . 1.346 gm. substance in 100 cc.  $\alpha$ ,  $-0.46^\circ$ .

$$[\alpha]_D^{25} = \frac{-0.46 \times 100}{2 \times 1.346} = -17.08^\circ.$$

*B. Optical Rotation of the Sodium Salt.*—0.673 gm. of acid was dissolved in a small amount of  $\text{H}_2\text{O}$ , 0.1 N NaOH added until just alkaline to phenolphthalein, and the color discharged with 2 drops of 0.1 N HCl. The volume was made to 50 cc.  $\alpha$ ,  $-0.40^\circ$ .

$$[\alpha]_D^{25} = \frac{-0.40 \times 100}{2 \times 1.346} = -14.86^\circ.$$

*Reducing Action.*—The acid quickly decolorizes neutral  $\text{KMnO}_4$  in the cold, but it does not reduce hot alkaline copper reagents. When boiled for a short time with dilute HCl it becomes an active reducing substance as does the ester.

#### *Anhydroglucose Cycloacetoacetic Acid.*

*Preparation.*—Glucose cycloacetoacetic acid (4.5 gm.) was dissolved in 50 cc. of  $\text{H}_2\text{O}$  and boiled under reflux for 6.5 hours. The solution became quite yellow and upon cooling no crystals

separated. The solution was concentrated to a small volume and placed in the cold room (approximately 0°C.). Rosettes of needle-shaped crystals formed. The crystals were separated and washed on the suction filter with a very small amount of cold water, and recrystallized from a small volume of hot water. After drying the compound melted at 140°C. Yield about 2 gm. 7.9 gm. of acid dissolve in 100 cc. of H<sub>2</sub>O at 20°C.

*Analysis.* 0.1528, 0.1816 gm. substance: CO<sub>2</sub> 0.2950, 0.3502, H<sub>2</sub>O 0.0726, 0.0861.

Calculated for C<sub>10</sub>H<sub>12</sub>O<sub>6</sub>. C 52.59, H 5.30.

Found. " 52.51, 52.45, H 5.27, 5.26.

0.2168 gm. substance: 9.54 cc. 0.1 N NaOH. Neutral equivalent 227.25; calculated 228.14.

*Hydroxyl Groups.*<sup>4</sup>—0.3196 gm. of acid was placed in a ground glass-stoppered bottle with 10 cc. of pyridine and 2 cc. of acetic anhydride. A blank was prepared also. The bottles were heated in an oven at 52°C. for 16 hours. The products were cooled to almost 0°C., 200 cc. of ice water added, and titrated with 0.5125 N NaOH to almost the end-point of phenolphthalein and the titration completed with 0.1 N NaOH. Blank, 81.30 cc. of 0.5125 N NaOH. 0.5125 N NaOH for acetylated acid 78.43 cc. 0.5125 N NaOH to neutralize 0.3196 gm. of acid, 2.72 cc. 0.5125 N NaOH equivalent to acetyl bound by hydroxyl 2.87 + 2.72 = 5.59 cc. 1 cc. 0.5125 N NaOH = 0.022 gm. CH<sub>3</sub>CO. Acetyl found, 0.022 × 5.59 = 0.123 gm. Calculated for two hydroxyl groups, 0.120 gm.

#### A. Optical Rotation of the Acid.

1. Solvent CH<sub>3</sub>OH. 1.248 gm. substance in 100 cc. α, - 2.79°.

$$[\alpha]_D^{25} = \frac{-2.79 \times 100}{2 \times 1.248} = -111.7^\circ.$$

2. Solvent H<sub>2</sub>O. 1.248 gm. substance in 100 cc. α, - 3.0°.

$$[\alpha]_D^{25} = \frac{-3.0 \times 100}{2 \times 1.248} = -120.1^\circ.$$

<sup>4</sup> This method of determining hydroxyl groups is discussed in a paper by Peterson, V. L., and West, E. S., *J. Biol. Chem.*, 1927, lxxiv, 379.

*B. Optical Rotation of the Sodium Salt.*—Solvent  $\text{H}_2\text{O}$ . 0.624 gm. of acid was dissolved in  $\text{H}_2\text{O}$  and  $\text{NaOH}$  added until just alkaline to phenolphthalein. Color discharged with a trace of 0.1 N  $\text{HCl}$ . Volume made to 50 cc.  $\alpha$ ,  $-3.16^\circ$ .

$$[\alpha]_D^{25} = \frac{-3.16 \times 100}{2 \times 1.248} = -126.6^\circ.$$

*Reducing Action.*—The acid quickly decolorizes  $\text{KMnO}_4$  in aqueous solution in the cold, but does not reduce hot alkaline copper reagents. When heated for a short time with dilute  $\text{HCl}$  it becomes powerfully reducing just as glucose cycloacetoacetic acid, and similarly the reducing action on alkaline copper reagents is destroyed by warming with alkali.

*Tetraacetylglucose Cycloacetoacetic Acid.*

*Preparation.*—Glucose cycloacetoacetic acid (10 gm.), pyridine (50 cc.), and acetic anhydride (30 gm.) were mixed and allowed to react at  $37^\circ\text{C}$ . for several days. The product was poured into  $\text{H}_2\text{O}$ , chloroform added, acidified with  $\text{H}_3\text{PO}_4$ , agitated, the  $\text{CHCl}_3$  solution separated, washed thoroughly with saturated  $\text{NaCl}$  solution and  $\text{H}_2\text{O}$ , and dried over  $\text{Na}_2\text{SO}_4$ . The chloroform was removed under a vacuum, leaving a heavy syrup which did not crystallize. The syrup was dissolved in hot dilute alcohol and upon standing an oil separated. In a few days oval clusters of needle-shaped crystals formed in the surface of the liquid, and after 10 days all of the oil had crystallized. The crystals were filtered off under suction and washed with a small amount of  $\text{H}_2\text{O}$  and dried. M.p.  $94^\circ\text{C}$ .; yield 1 gm.

*Analysis.* 0.3169 gm. substance: 7.61 cc. 0.1 N  $\text{NaOH}$ . Neutral equivalent 416.2; calculated 414.

*Optical Rotation.*—Solvent  $\text{CHCl}_3$ . 1.6 gm. substance in 100 cc.  $\alpha$ ,  $-1.23^\circ$ .

$$[\alpha]_D^{25} = \frac{-1.23 \times 100}{1.6 \times 2} = -38.4^\circ.$$

*Reducing Action.*—The acid decolorizes aqueous  $\text{KMnO}_4$  in the cold.

*Tetramethylglucose Cycloacetoacetic Acid.*

Glucose cycloacetoacetic acid was methylated essentially according to the procedure of Haworth (16). Glucose cyclo-

acetoacetic ester (25 gm.) was dissolved by heating with 50 cc. of 3 N NaOH. The solution was placed in a 2 liter round bottom flask fitted with a mercury-sealed stirrer, two burettes, and a reflux water condenser through a rubber stopper. The apparatus was placed in a H<sub>2</sub>O bath at 70°C. During the course of an hour dimethyl sulfate (141 cc.) and 55 per cent NaOH (240 cc.) were permitted to flow slowly into the reaction flask from the burettes with constant stirring. Care was taken to add the alkali at such a rate that the contents of the flask were kept distinctly alkaline at all times. The water bath was then heated to boiling for an hour and the product permitted to stand overnight. A large amount of sodium sulfate separated. 100 cc. of chloroform were added to the product, and while being kept cold, it was acidified to methyl orange with H<sub>3</sub>PO<sub>4</sub>. After thorough agitation the CHCl<sub>3</sub> layer was separated. The solution was extracted a second time with chloroform and the combined extracts washed thoroughly with saturated NaCl solution, dried with Na<sub>2</sub>SO<sub>4</sub>, and the CHCl<sub>3</sub> removed under a vacuum at 100°C. The heavy oil did not crystallize. It gave a neutral equivalent of 296.8, corresponding to a mixture of tri- and tetramethylated acids. It was dissolved in 50 cc. of 5 N NaOH and subjected to a second methylation as above, dimethyl sulfate (55 cc.) and 55 per cent NaOH (95 cc.) being used. The very heavy oil obtained after removal of the CHCl<sub>3</sub> was distilled in a vacuum. It passed over as a light yellow exceedingly heavy syrup at 205°C., 0.9 mm. Yield 8 gm. After standing overnight a large rosette of needles had formed within the syrup. The mistake of stirring the mass was made, whereupon it quickly solidified to a very hard semicrystalline mass. As yet no satisfactory procedure for recrystallization from a solvent has been found and consequently no melting point data are available. Work on this point is in progress. The compound is difficultly soluble in cold water. When heated with water it slowly goes into solution but suffers hydrolysis of the acetoacetic acid ring as explained below. It is readily soluble in the common organic solvents.

*Analysis.* 0.1932, 0.1676 gm. substance: CO<sub>2</sub> 0.3926, 0.3430, H<sub>2</sub>O 0.1272, 0.1101.

Calculated for C<sub>14</sub>H<sub>22</sub>O<sub>7</sub>. C 55.62, H 7.28.

Found. " 55.41, 55.75, H 7.31, 7.29.

0.4411 gm. substance in dilute alcohol, 14.58 cc. 0.1 N NaOH. Neutral equivalent 302.54; calculated 302.

Methoxyl (Zeisel). 0.1143, 0.0899 gm. substance: AgI 0.3488, 0.2795, OCH<sub>3</sub> 0.04604, 0.03688. Calculated 41.06; found, 40.28, 41.03.

*Optical Rotation.*—Solvent CHCl<sub>3</sub>. 2.554 gm. substance in 100 cc. α, -2.16°.

$$[\alpha]_D^{25} = \frac{-2.16 \times 100}{2 \times 2.554} = -42.2^\circ.$$

*Reducing Action.*—The compound does not reduce alkaline copper solutions but instantly decolorizes aqueous permanganate in the cold.

#### *Tetramethylglucose Acetoacetic Acid.*

Though the compound has not as yet been isolated in the pure condition, preliminary evidence indicates that it has been obtained in solution but admixed with methylated glucose cycloacetoacetic acid. In early attempts to prepare tetramethylglucose cycloacetoacetic acid the alkaline reaction product was acidified with HCl instead of H<sub>3</sub>PO<sub>4</sub> and without care to keep the temperature down. The methylated syrup obtained possessed strong reducing action toward alkaline copper reagents, gave a characteristic nitroprusside reaction, and when treated with I<sub>2</sub> and NaOH iodoform precipitated instantly. All of these reactions correspond to those of glucose cycloacetoacetic acid and its ester after boiling with dilute HCl, and indicate the presence of a very reactive CH<sub>3</sub>CO group formed by hydrolysis of the carbon ring involving the acetoacetic acid chain. A sample of methylated acid (one methylation) prepared with HCl when titrated in dilute alcohol, cold, showed a neutral equivalent of 297.3 (calculated 302), but when an excess of 0.1 N NaOH was added to the acid, heated, and back titrated with 0.1 N HCl the neutral equivalent was 260.6. That the alkali had not split CO<sub>2</sub> from the substance was proved by an experiment in which it was heated 2.5 hours in 1.4 N NaOH. No CO<sub>2</sub> was liberated by this treatment. Also the end-point to phenolphthalein in the titration of the alkaline solution of the acid was sharp. The titrated solution was acidified with H<sub>3</sub>PO<sub>4</sub> and steam-distilled. The distillate contained 2 to 3 cc. of 0.1 N volatile acid. These facts indicate that probably the hot alkali split off the CH<sub>3</sub>CO group of the open ring compound as acetic



acid accordingly decreasing the neutral equivalent. More direct evidence for the rupture of the carbon ring of tetramethylglucose cycloacetoacetic acid and the formation of tetramethylglucose acetoacetic acid was obtained in the following manner. Approximately 1 gm. of tetramethylglucose cycloacetoacetic acid was placed in a flask with 25 cc. of  $H_2O$  and boiled under a reflux for 4 hours. A yellowish solution was obtained which possessed a sour, burning taste. This solution, made alkaline with 0.5 N NaOH, gave a deep red coloration. Benedict's copper reagent was reduced practically *instantly in the cold* when added to the alkaline solution. With Benedict's reagent added to the acid solution *without preliminary neutralization*, reduction took place *in the cold* in several minutes. The solution treated with  $I_2$  and NaOH *instantly* gave a copious precipitate of iodoform.

Further work on the preparation of the substance in the pure condition and a study of its properties both chemical and physiological is in progress.

*Anhydroglucose Cycloacetoacetic Ester.*

*Preparation.*—Glucose cycloacetoacetic ester (25 gm.) and concentrated HCl (100 cc.), cooled to  $0^\circ C.$ , were mixed and allowed to stand 5 minutes. The solution was diluted to 300 cc. with cold  $H_2O$  in a large flask, neutralized with solid  $NaHCO_3$ , and the precipitated heavy oil taken up in chloroform. The extract was washed with saturated NaCl solution, dried with  $Na_2SO_4$ , and the chloroform distilled off. The heavy oil distilled without decomposition at  $205^\circ C.$ , 0.8 mm. Yield 14.5 gm. Samples of the product have stood many months with no trace of crystallization. The compound generally shows a faint reducing action toward hot alkaline copper reagents due to the presence of a small amount of the isomer dianhydroglucose acetoacetic ester, though preparations have been obtained which were exceptions.

*Analysis.* 1. Preparation freed of solvent and dried. Not vacuum-distilled. 0.2767 gm. substance:  $CO_2$  0.5568,  $H_2O$  0.1520. Found, C 54.87, H 6.10.

2. Preparation vacuum-distilled. 0.2720 gm. substance:  $CO_2$  0.5581,  $H_2O$  0.1523.

Calculated for  $C_{12}H_{16}O_6$ . C 56.23, H 6.29.  
Found. " 55.95, " 6.22.

*Optical Rotation.*—Solvent  $\text{CH}_3\text{OH}$ . 1.401 gm. substance in 100 cc.  $\alpha$ ,  $-2.523^\circ$ .

$$[\alpha]_D^{20} = \frac{-2.52 \times 100}{2 \times 1.401} = -89.9^\circ.$$

Three other preparations showed  $[\alpha]_D$  values of  $-83.1^\circ$ ,  $-88.1^\circ$ , and  $-87.4^\circ$ . The first of these showed no reducing action on alkaline copper reagents. It may have contained a small amount of glucose cycloacetoacetic ester. This would have caused a lowered rotation.

*Saponification of Anhydroglucose Cycloacetoacetic Ester. Anhydroglucose Cycloacetoacetic Acid.*

Anhydroglucose cycloacetoacetic ester (3 gm.) was treated with 10 per cent  $\text{NaOH}$  (10 cc.) and allowed to stand at room temperature overnight. The solution became slightly brown in color. It was thoroughly cooled, acidified to methyl orange with  $\text{HCl}$ , and seeded with anhydroglucose cycloacetoacetic acid obtained by boiling glucose cycloacetoacetic acid in aqueous solution as described previously. Soon the liquid set to a firm mass of crystals. These were separated, washed with a little cold  $\text{H}_2\text{O}$ , and recrystallized from hot water. When dried in a vacuum over  $\text{H}_2\text{SO}_4$  the crystals melted at  $140.5^\circ\text{C}$ . and mixed with anhydroglucose cycloacetoacetic acid obtained by boiling glucose cycloacetoacetic acid solution, melted at  $141^\circ\text{C}$ . These acids are accordingly identical, and the loss of water when glucose cycloacetoacetic acid is heated in aqueous solution and when the ester is treated with concentrated  $\text{HCl}$  in the cold involves the same hydroxyl groups of the glucose chain.

*Diacetylanhydroglucose Cycloacetoacetic Ester.*

*Preparation.*—Anhydroglucose cycloacetoacetic ester (17 gm.) was mixed with acetic anhydride (40 gm.) and pyridine (50 gm.). Reaction quickly took place with evolution of heat. The mixture was placed in the warm room at  $36^\circ\text{C}$ . for 44 hours. The brown solution was poured upon cracked ice and stirred. A very heavy oil separated which failed to crystallize. It was separated and mixed with  $\text{CO}_2$  snow but no crystallization could be induced. The oil was then taken up in  $\text{CHCl}_3$ , washed, dried with  $\text{Na}_2\text{SO}_4$ ,

and the solvent removed. The syrup distilled at 175°C., 0.6 mm., without decomposition, as a golden yellow oil. CO<sub>2</sub> snow was added but crystallization did not take place. The oil was redistilled for analysis. Yield 13.9 gm. The compound does not reduce hot alkaline copper solutions.

*Analysis.* 0.2828 gm. substance: CO<sub>2</sub> 0.5829, H<sub>2</sub>O 0.1503.

Calculated for C<sub>16</sub>H<sub>20</sub>O<sub>8</sub>. C 56.47, H 5.88.

Found. " 56.20, " 5.90.

Acetyl. 1.0526 gm. substance, hydrolyzed with 30 per cent H<sub>3</sub>PO<sub>4</sub>. Cc. 0.1 N acetic acid calculated, 61.91; found, 62.36.

*Optical Rotation.*—Solvent CHCl<sub>3</sub>. 1.522 gm. substance in 100 cc. α, -2.04°.

$$[\alpha]_D^{20} = \frac{-2.04 \times 100}{2 \times 1.522} = -67^\circ.$$

#### *Dianhydroglucose Acetoacetic Ester.*

*Preparation.*—Glucose cycloacetoacetic ester (10 gm.) and concentrated HCl (20 cc.) were mixed and allowed to stand 35 minutes at 30°C. The ester dissolved and the solution became brown. It was poured into 150 cc. of H<sub>2</sub>O and the acid neutralized with precipitated CaCO<sub>3</sub>. The heavy oil which separated was thoroughly extracted with chloroform, the extract washed with saturated NaCl solution, and dried with Na<sub>2</sub>SO<sub>4</sub>. After removal of the solvent the substance distilled as a light yellow oil at 200°C., 0.8 mm. Yield 3 gm. Many preparations were made under varying conditions of temperature, time, and proportions of reagents. If too much acid is used or the time of action too long or the temperature too high extensive decomposition and resinification take place. On the other hand, if the action is interrupted too quickly a considerable proportion of the isomeric oil, anhydroglucose cycloacetoacetic ester, is obtained, and, as yet, no way of effecting a separation has been found. The optical rotations of different samples cited below indicate that while the substance may analyze correctly for C and H, ethoxyl, and hydroxyl, yet varying amounts of the isomeric oil with a higher rotation are present, depending upon the time of action of HCl and other factors.

#### *Analysis.*

1. Preparation dried in a vacuum over H<sub>2</sub>SO<sub>4</sub> and not distilled. 0.2985 gm. substance: CO<sub>2</sub> 0.5927, H<sub>2</sub>O 0.1669. Found, C 54.1, H 6.21.

2. Preparation distilled. 0.2180, 0.2298 gm. substance: CO<sub>2</sub> 0.4484, 0.4746, H<sub>2</sub>O 0.1163, 0.1253.

Calculated for C<sub>12</sub>H<sub>14</sub>O<sub>6</sub>. C 56.23, H 6.29.

Found.

" 56.09, 56.27, H 5.92, 6.05.

Ethoxyl (Zeisel). AgI was fused with Na<sub>2</sub>CO<sub>3</sub>-K<sub>2</sub>CO<sub>3</sub> mixture, dissolved, acidified with H<sub>2</sub>SO<sub>4</sub>, Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> added, and the iodine distilled into KI and titrated. 0.3500 gm. substance: 13.38 cc. 1 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Weight of ethoxyl  $0.0045 \times 13.38 = 0.0601$ . Calculated for C<sub>12</sub>H<sub>14</sub>O<sub>6</sub>, 17.56; Found, 17.17.

Optical Rotation.—Solvent CH<sub>3</sub>OH. 1.401 gm. substance in 100 cc. α, -0.86°.

$$[\alpha]_D^{25} = \frac{-0.86 \times 100}{2 \times 1.401} = -30.6^\circ.$$

Another product which probably contained a very considerable amount of the isomer anhydroglucose cycloacetoacetic ester, gave  $[\alpha]_D^{25} = -45.7^\circ$ . Other values obtained for different samples were -42.8°, -50.0°.

Mr. Wm. G. Barrett has kindly analyzed several samples of the substance for the carbonyl group by the method of Ardagh and Williams (17) and found phenylhydrazine used up corresponding to about 20 per cent of the theory. This is very likely much too low because of the slight solubility of the phenylhydrazone in petroleum ether. It definitely indicates, however, the presence of a reactive carbonyl group.

The compound is soluble in dilute NaOH solution. When its alkaline solution is boiled or allowed to stand at ordinary temperature for a time profound resinification takes place. If this alkaline digest is cooled and acidified a large amount of tarry material separates. The action of acid upon the compound is similar to that of alkali. The compound reduces Fehling's solution *quickly in the cold*. It also reduces anthraquinone 1,5-disodium and 2-sodium sulfonates as well as methylene blue *in the cold* when dissolved in 0.1 N NaOH. It gives oily substances when treated with phenylhydrazine and *p*-nitrophenylhydrazine which rather quickly go to pieces and become black and tarry.

An alkaline solution of the compound gives an immediate precipitate of iodoform when treated with iodine. It also gives a fleeting red coloration with sodium nitroprusside which becomes greenish blue upon acidification with glacial acetic acid. The substance is not identical with the material showing reducing properties and giving the reactions of the CH<sub>3</sub>CO group obtained

by boiling glucose cycloacetoacetic ester with dilute HCl described above as shown by the observations that it only slowly loses its reducing power upon boiling with dilute alkali, and undergoes marked decomposition and resinification.

The compound does not lose an appreciable amount of CO<sub>2</sub> upon heating with dilute alkali. It is likely that acid decomposition takes place with the formation of CH<sub>3</sub>COOH by the splitting off of CH<sub>3</sub>CO as has been suggested in case of tetramethyl glucose acetoacetic acid.

*Diacetyldianhydroglucose Acetoacetic Ester.*

*Preparation.*—The compound was prepared exactly as its isomer, diacetylanhydroglucose cycloacetoacetic ester described above. It distilled in a vacuum (0.8 mm.) as a clear yellow oil at 220°C. 6 gm. acetylated product were obtained from 8 gm. dianhydroglucose acetoacetic ester.

*Analysis.* 0.3325 gm. substance: CO<sub>2</sub> 0.6892, H<sub>2</sub>O 0.1754.

Calculated for C<sub>16</sub>H<sub>20</sub>O<sub>8</sub>. C 56.47, H 5.88.

Found. " 56.52, " 5.86.

Acetyl. 0.5452 gm. substance, hydrolyzed with 30 per cent H<sub>3</sub>PO<sub>4</sub>. Cc. 0.1 N acetic acid calculated, 32.0; found, 31.3.

*Optical Rotation.*—Solvent CHCl<sub>3</sub>. 1.562 gm. substance in 100 cc. α, -1.86°.

$$[\alpha]_D^{25} = \frac{-1.86 \times 100}{2 \times 1.562} = -59.5^\circ.$$

The compound reduces hot alkaline copper reagents and gives the various reactions of the CH<sub>3</sub>CO group given by the non-acetylated ester. This suggests that both hydroxyl groups are located in the glucose chain, and that the enolic hydroxyl of the acetoacetic acid chain was not acetylated.

*Trianhydroglucose Acetoacetic Ester.*

*Preparation.*—It was early noted that in old preparations of distilled dianhydroglucose acetoacetic ester fine yellow crystals appeared in small quantities. With certain variations of preparations as for instance, longer action of HCl, a considerable amount of these crystals separated, sometimes while distillation was in progress. It was found that the crystals could be easily

separated from the dianhydro compound by treatment with approximately 70 per cent alcohol in the cold, in which the crystals are insoluble. After separation and washing with dilute alcohol the crystals were dissolved in a small amount of pyridine and precipitated by the addition of  $\frac{1}{2}$  to 1 volume of  $H_2O$ . The crystals were separated and washed with dilute alcohol under suction. The compound, dried in a vacuum over  $H_2SO_4$ , melted at  $137^\circ C$ . From 25 gm. of dianhydroglucose acetoacetic ester 1.2 gm. of purified material were obtained. When examined under the microscope the substance was seen to be composed of very small yellow crystals in the form of sheaves. That the compound is formed from dianhydroglucose acetoacetic ester in the process of distillation is indicated by the observations that if the crystals be separated from the oil as outlined above and the oil then redistilled a second crop of crystals is obtained. Also a preparation of dianhydroglucose acetoacetic ester, dried and freed from solvent under high vacuum and allowed to stand a few days, gave no crystals. When the syrup was vacuum-distilled crystals promptly separated.

*Analysis.* 0.1475, 0.1533 gm. substance:  $CO_2$  0.3270, 0.3403,  $H_2O$  0.0792, 0.0814.

Calculated for  $C_{12}H_{14}O_6$ . C 60.50, H 5.88.

Found. " 60.45, 60.53, H 5.96, 5.89.

Ethoxyl (Zeisel). 0.1828 gm. substance: AgI 0.1742, ethoxyl 0.0334. Calculated for one  $C_2H_5O$  group, 18.92; found, 18.26.

Hydroxyl was estimated by the pyridine acetic anhydride titration method described above under anhydroglucose cycloacetoacetic acid. 0.3285 gm. substance. Blank titration 81.30 cc. 0.4925 N NaOH; sample titration 78.56 cc. 0.4925 N NaOH. Cc. 0.4925 N NaOH equivalent to  $CH_3CO$  bound by the compound,  $81.30 - 78.56 = 2.74$ . Weight of acetyl calculated for one hydroxyl group, 0.0593; found, 0.0581.

The compound is optically inactive. The acetylated compound was easily hydrolyzed as shown by the addition of an excess of alkali after the titration was completed, the indicator being decolorized in a short time.

The compound is insoluble in water, readily soluble in dilute alkali and pyridine, and sparingly soluble in alcohol. It is not

resinified and decomposed by boiling alkali as is dianhydroglucose acetoacetic ester. It does not reduce hot alkaline copper reagents or Tollens' reagent but reduces aqueous permanganate in the cold. With iodine and alkali it gives quickly a precipitate of iodoform. Phenylhydrazine reacts with it in alcoholic solution to form a yellow crystalline derivative which is described below.

*Phenylhydrazine Derivative of Trianhydroglucose Acetoacetic Ester.*

*Preparation.*—Trianhydroglucose acetoacetic ester was heated for a short time with phenylhydrazine and a little acetic acid in alcohol. Upon cooling rosettes of yellow needle-shaped crystals separated. A quantity sufficient for analysis was obtained by refluxing the dilute alcoholic solution of the syrup (25 gm.) separated from dianhydroglucose acetoacetic ester with phenylhydrazine (10 gm.), sodium acetate (5 gm.), and acetic acid (8 gm.). This was possible because the separation of the trianhydro compound by alcohol treatment was incomplete. The material was dissolved in hot pyridine and precipitated by dilution with  $H_2O$ . The crystals were filtered off, washed thoroughly with dilute alcohol, and dried over  $H_2SO_4$  in a vacuum. M.p.  $177^\circ C$ . when heated slowly,  $180^\circ C$ . when heated rapidly (with decomposition). The compound is insoluble in alkali.

*Analysis.* 0.1138, 0.1492 gm. substance:  $CO_2$  0.2820, 0.3705,  $H_2O$  0.0694, 0.0896.

Theoretical for:

$C_{18}H_{20}O_4N_2$ . C 65.75, H 6.13.

$C_{18}H_{18}O_3N_2$ . " 69.50, " 5.84.

$C_{17}H_{20}O_3N_2$ . " 67.96, " 6.68.

Found. C 67.57, 67.72, H 6.77, 6.67.

Nitrogen (Kjeldahl using glucose) (18). 0.1023, 0.1067, 0.1073 gm. substance: cc. 0.1 N HCl, 6.79, 7.00, 7.04.

Calculated for  $C_{17}H_{20}O_3N_2$ . N 9.33.

Found. N 9.29, 9.19, 9.14.

Ethoxyl (Zeisel). 0.1110 gm. substance: AgI 0.0827, ethoxyl 0.01584. Calculated for  $C_{17}H_{20}O_3N_2$ , one  $C_2H_5O$  group, 14.97; found, 14.27.

The formula  $C_{18}H_{20}O_4N_2$  represents the monohydrazone of trianhydroglucose acetoacetic ester,  $C_{18}H_{18}O_3N_2$  this hydrazone minus  $H_2O$ , and  $C_{17}H_{20}O_3N_2$  the hydrazone minus CO.

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#### SUMMARY.

Glucose reacts with acetoacetic ester in the presence of zinc chloride to form a condensation product, glucose cycloacetoacetic ester. From this substance a series of derivatives has been obtained including glucose cycloacetoacetic acid, anhydroglucose cycloacetoacetic ester and acid, dianhydroglucose acetoacetic ester, trianhydroglucose acetoacetic ester, and various acetylated and methylated derivatives. Some of these substances are powerful reducing agents.

The compounds are discussed with respect to their possible relationship to the phenomenon of antiketogenesis.

A theory of catalytic and enzymic action based upon electronic concepts is advanced.

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# THE FUNDAMENTAL FOOD REQUIREMENTS FOR THE GROWTH OF THE RAT.

## I. GROWTH ON A SIMPLE DIET OF PURIFIED NUTRIENTS.\*

BY LEROY S. PALMER AND CORNELIA KENNEDY.

(From the Section of Animal Nutrition, Division of Agricultural Biochemistry, University of Minnesota, St. Paul.)

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### INTRODUCTION.

The advances in the knowledge of nutrition during the past three decades have led to the adoption of the view that the essential food requirements for growth of the rat can be expressed in terms of energy, biologically adequate protein, mineral salts, and the already known vitamins. McCollum (1) expressed the view of this matter which prevailed until very recently in the statement that for the rat: "A single purified protein, a source of the sugar glucose, nine mineral elements and two uncharacterized dietary factors, . . . . . [are] entirely adequate for the support of growth and prolonged well-being."

The uncharacterized dietary factors implied in this formula are those now generally recognized as vitamins A and B. Of the other vitamins and vitamin-like substances whose existence has been proved or indicated since McCollum expressed the above view, very few workers regard either vitamin C<sup>1</sup> or bios as necessary to be supplied in the diet of growing rats. Some will perhaps regard the antirachitic factor, vitamin D or its biological antecedent,

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<sup>1</sup> Harden, A., and Zilva, S. S., (*Biochem. J.*, 1918, xii, 408) and Drummond, J. C., (*Biochem. J.*, 1919, xiii, 77) conclude that rats make more favorable growth with antiscorbutic vitamin than without, but as the evidence is based on the use of fruit juices containing vitamin A or B or both, it is not possible to decide that the favorable result noted was due exclusively to vitamin C.

radiant energy, as necessary for growth and well being; but the proof of the necessity of this factor is inconclusive so long as vitamin A is abundant and the other dietary requirements are met and the various dietary constituents properly balanced.<sup>2</sup>

The formula, also, must not be interpreted as implying adequacy for all the functions of the mature rat, such as reproduction and lactation; the former requires vitamin E. As for lactation some have postulated the existence of a special factor and others the need for special mineral elements.

With the above reservations or additions and the substitution of the words "apparent well being" for the word "well being," it may safely be said that the great majority of nutrition workers will accept McCollum's older formula for growth as fulfilling the requirements indicated by our present knowledge of the subject. However, if an attempt is made to interpret such a formula in terms of chemically pure substances, several obstacles at once arise. In the first place, the known vitamins required for growth are not yet attainable as pure chemical entities. In the second place, it is not as yet demonstrated that successful growth ensues when the organic nutrients such as protein and carbohydrate are limited to single *pure* chemical substances and the vitamins are supplied even in as concentrated and pure a form as possible. In other words, the fact cannot be lost sight of that the term "pure" is relative when applied to substances of biological origin. When applied to nutritive substances and nutritive formulæ so far employed, the term cannot be applied too rigidly. *No nutritive régime of any kind has yet been attained with pure chemical substances.*

In spite of these facts which are undoubtedly obvious to every student of nutrition, the measure of success attained in simple growth experiments using purified nutrients and more or less impure sources of vitamins A and B seems to support the belief that growth

<sup>2</sup> The conclusion of Steenbock, H., Nelson, M. T., and Black, A., (*J. Biol. Chem.*, 1924, lxii, 275) is accepted that growth failure on certain diets cannot be interpreted as due to vitamin A deficiency unless the anti-rachitic factor is supplied. However, this conclusion cannot as yet be regarded as carrying with it the implication that both vitamin A and the antirachitic factor are required for growth under all conditions; otherwise the literature would not abound in successful growth experiments in which there is no apparent indication of the antirachitic factor being supplied.

of the rat is determined by known, or at least attainable substances of known existence.

#### HISTORICAL.

Lack of space forbids both the citation and detailed review of all the published experiments which appear to support this belief. Typical instances only will be cited and comment limited to details which detract from the acceptance of the results as having proved the possibility of growth on the above formula. Mention will also be made of experiments which have been interpreted as indicating the need of other basic nutrients for growth.

In the growth experiments of McCollum and Davis (2, 3), the rats all had access to their feces, and several of the important ingredients of the rations, such as casein and lactose, were of doubtful purity from the standpoint of organic substances of unknown nature. Only eleven rats in all were reported, and the maximum duration of any experiment was 6 months.

The successful growth of rats reported by Funk and Macallum (4) on a synthetic diet was limited to 30 days. The diet contained commercial starch and sugar and unrendered butter. The animals, however, did not have access to their feces (5).

Osborne and Mendel have reported both long continued (6, 7) and short periods (8) of normal growth on synthetic diets, but in all cases feces were available *ad libitum*. Alcohol was the only solvent used to remove impurities of organic nature from the proteins employed. In the long continued experiments whole yeast was fed and one diet contained lactose in large amounts, but nothing is stated regarding its purity or purification. In the short experiment, protein-free yeast fraction replaced the whole yeast formerly employed.

Drummond (9) does not give the methods employed for purifying the ingredients of the synthetic diets which gave four generations (10) of normal animals, but feces were available to the animals. High percentages of yeast or marmite were employed and in the experiment of four generations lemon juice was also fed.

In the successful growth experiments of Heller (11), whole yeast made up 5 per cent of the diet, and the rats were kept on sawdust or shavings. The protein was extracted with water and alcohol.

The synthetic diets used by Steenbock, Sell, and Nelson (12) for normal growth contained whole yeast or ether-extracted wheat embryo, and the casein was a commercial product merely leached with acid water.

In our (13) own successful growth experiments already published, the rats had access to their feces. A considerable proportion of the diet was whole yeast although success was reported also with use of the alcohol extract of ether-extracted wheat embryo equivalent to 15 per cent of the fat-free embryo in the diet. The casein purification (14) involved the use of water, alcohol, and ether.

The foregoing experiments have been interpreted as indicating that the requirements for growth of rats can be met by a simple mixture of known or attainable substances.

The question may well be raised, however, as to what the standard of growth should be in measuring success in nutrition experiments of this character. Most investigators using rats, seem to be satisfied if their animals attain the Donaldson average or even if they show steady and fairly rapid growth during the first few weeks or months after weaning. It may be remarked, however, that it will not be possible to claim real success in experiments with purified nutrients until the experimental animals attain the optimum as frequently as is experienced among breeding stock fed a mixed diet of natural foods. No concrete evidence has been submitted that this is possible, even on the types of purified diets reviewed in the foregoing paragraphs.

Before presenting our own experience in testing this hypothesis under more rigid conditions than we or others have so far reported, it may be well to mention several experiments which have been interpreted as indicating that the vitamin requirements for growth are not met solely by those furnished by liberal amounts of butter fat, wheat embryo extract, yeast, or yeast fractions.

According to Emmett and Luros (15, 16) milk contains a water-soluble, heat-labile, growth-promoting vitamin other than vitamin B. Lactose, extracted with hot 95 per cent alcohol, carried the new vitamin in their experiments.

Funk, Paton, and Freedman (17) found that casein purified by treating a solution of sodium caseinate with  $H_2O_2$  and fullers' earth, loses considerable growth-promoting value which was not

completely restored by such quantities of vitamins A, B, or C as were supplied in the form of butter fat, vitamin B (Harris), and orange juice. The supplemented diet was too low in vitamin A, however, to insure optimum growth. The evidence would be more convincing had cod liver oil been employed to furnish both vitamin A and vitamin D.

In the discussion of their data, Funk, Paton, and Freedman indirectly suggest a possible relationship between the substances removed from casein and an antipellagra vitamin; but it is obvious that this could not have been the antipellagra vitamin which Goldberger and Tanner (18) found in vitamin B (Harris) because Funk used this product as his source of vitamin B.

Smith and Hendrick (19) and Goldberger and coworkers (20) have shown in experiments of 3 to 4 weeks duration, that autoclaved yeast comprising 8 to 10 per cent of the diet supplies a necessary growth-promoting factor for the rat, not supplied by Seidell's purified vitamin B or by alcoholic extracts of corn-meal, when the diet is otherwise presumably complete and of the usual synthetic type. These experiments resemble those of Emmett and Luros (21) who found that yeast and unmilled rice retained their growth-promoting power for rats even after 6 hours heating at 120°C., although the antineuritic effect for pigeons was lost after 2 to 3 hours heating.

These newer findings regarding growth-promoting requirements of the rat suggest that vitamin B will eventually be fractionated into more than one growth-promoting substance, only a portion of which carries the antineuritic properties.

#### EXPERIMENTAL.

*General Purpose and Plan of Experiments.*—The experiments reported in this paper developed out of an attempt to determine how many generations of rats could be secured on what appears to be an adequate synthetic diet of purified nutrients. The experiments were begun in July, 1922. We are presenting both the original experiment and our subsequent experience with the same ration in order to show that the outcome has been invariably the same even though there have been certain changes in technique, environment, etc., to which the results might otherwise be attributed.

The original experiment was prompted by the fact that we felt that there were insufficient data to support the belief that an adequate diet could be expressed in terms of known nutrient substances, particularly when judged by the standard (22) that satisfactory nutrition can be measured only by "normal growth to the normal adult size, at about the usual rate," and when there is also "normal reproduction and suckling of the young, and repetition of this at the normal intervals." Our feeling regarding the inadequacy of nutrition knowledge as judged by this standard was augmented by certain rather contradictory experiences encountered in the course of other work carried on in this laboratory, most of which has been published (13, 14). In one experiment (13) we secured good growth and some reproduction (three litters) but no rearing of young on a synthetic diet using butter fat as source of vitamin A and wheat embryo extract as source of vitamin B. However, when fat from ewe's milk (14) was used as a source of vitamin A, not even growth was secured until a small amount (0.8 per cent) of yeast was incorporated in the diet; following this there was good growth, early reproduction, and the successful rearing of seven out of twelve young in two litters.

In planning our experiments we chose as our basal ration, one which had given good growth in a previous experiment (13). This ration which carries our laboratory designation, Ration 5, consists of purified casein, 18 parts, salt mixture (23), 3.7 parts, agar-agar, 2 parts, butter fat, 5 parts, dextrin to 100 parts, the latter carrying the alcohol extract of ether-extracted commercial wheat embryo equivalent to 15 parts of ether-extracted embryo.

*We wish to emphasize the fact that this ration does not consist solely of known chemical nutrients.* Based on our experience with yeast (13) as a source of vitamin B, we felt, however, that it contains fewer unknown substances than when yeast is used.

*Preparation of Ingredients of Ration.*—The various ingredients of the basal ration were prepared from time to time as needed. There were some modifications in the procedure of preparation during the course of the 4 years of experimentation. The basal ration itself underwent some modification. Although these deviations from the original had no effect on the outcome of the experiment so far as could be observed, it may be well to mention them briefly.

*Casein.*—Casein in all cases was prepared from raw milk skimmed by the centrifugal separator. The method was essentially the so called grain curd method (24), HCl being used as precipitating acid. The grainy curd was purified in all cases by successive leaching with (a) distilled water at pH = 4.8, (b) ethyl alcohol, and (c) ethyl ether, passing from one solvent to the next without intermediate drying. Following the ether purification the product was dried at a temperature not exceeding 65°C. and then pulverized. A snowy white powder always resulted. There have been numerous modifications in the details of this procedure. These changes have been for the purpose of making possible a more extended and more drastic purification. We were impressed by the fact that insufficient importance has been attached to the possibility that organic compounds of unknown and untested nutritive value may be adsorbed by casein in its separation from milk. We have attempted, therefore, to secure more nearly than has heretofore been attained a single pure protein for our basal ration.

No useful purpose will be served by enumerating our progress in purifying casein. It will be sufficient to give our present procedure. The freshly precipitated grain curd casein from several hundred pounds of separator milk is placed on a wire rack covered with cheese-cloth and thoroughly rinsed with distilled water at pH = 4.8. The casein is then suspended in a volume of distilled water approaching the original volume of milk, and ammonia water added until the casein redissolves on gentle stirring. When an excess of ammonia is present so that there is a faint odor of the alkali fumes, the casein solution is allowed to stand overnight in the vat in order to insure complete dispersion. The casein is reprecipitated from this solution by the grain curd method, the curd rinsed on the rack as before, and the rack containing the casein suspended in a large volume of distilled water at pH = 4.8. If one raises the temperature of this water to 80–90°C. by using the steam jacket of the cheese vat and maintains this temperature for 2 or 3 hours, it is possible to remove a very large amount of mineral salts as well as other impurities which make the water milky. This cooked curd is now either leached on the rack for 5 to 7 days in acidified distilled water (pH = 4.8), the water being changed twice daily, or is transferred to sacs each holding about 1 kilo of



moist curd and placed in a large percolator type of extractor.<sup>3</sup> Percolation with fresh distilled water is continued for 4 to 6 days, keeping the sacs of casein immersed in the water. In the leaching purification, there is little visible extraction after the first few changes of water, but in the percolation the amount of water-soluble impurity removed is seen to be very significant inasmuch as it is possible to concentrate it in the vaporizing column of the apparatus.

Following the water purification the casein curd is pressed as dry as possible in a cheese press, ground in a food chopper, replaced in the sacs, and extracted with alcohol. Alcohol of 60 to 70 per cent by volume is added to the percolator but the percolation itself is necessarily effected by somewhat stronger alcohol (about 85 to 90 per cent) due to the concentration of the alcohol in the vaporizing column during vaporization. The alcohol extraction is continued for about 7 days (24 hour days), the first few days with cold alcohol and finally with nearly boiling alcohol. The impurities are removed slowly by alcohol in this method so that it is unlikely that we continue the process long enough to free the casein completely from alcohol-soluble substances. This may account in part for the variable quantity of substance obtained in the extract which we find amounts to 3 to 5 per cent on the dry matter basis. The bulk of the alcohol-soluble material is the prolamin of milk, but there are also numerous substances of unknown nature.

The casein granules have a rather tough elastic nature following the alcohol extraction, and dry to very flinty particles which are reduced to powder with great difficulty if at all. To avoid this, and permit a more thorough ether extraction, the alcohol-moist casein is pressed or at times merely allowed to drain thoroughly in the percolation chamber, and without drying submitted to a 2 or 3 day continuous extraction with ether in the same manner that the water and alcohol extractions were made. The extraction apparatus delivers 300 to 350 cc. of fresh ether per minute which is a much greater stream than is obtained with either water or alcohol. This makes it possible to shorten the ether extraction period in comparison with the water or alcohol extraction. The

<sup>3</sup> We use the John Uri Lloyd patent drug extractor, holding about 10 kilos of material. This has been used by us since the summer of 1923.

amount of substance removed by ether is influenced greatly by the completeness with which the fat is removed from the original milk by the separator. In an average extraction it amounts to 1 to 3 per cent of the dry casein.

The completely extracted casein granules are spread in a shallow pan and placed in a steam-heated drying drawer through which a current of air passes. After several days the grains are pulverized in a mill, and are readily reduced to a snow-white dust.

*Mineral Salts.*—The mineral salt mixture was made in the usual manner from presumably pure laboratory reagents. As already indicated we used the McCollum mixture No. 185 (23). The practice has been to mix the salts together in about 4 kilo lots except for the iron citrate which is added to each 500 gm. portion as needed because of its catalytic effect in decomposing the calcium lactate. This salt mixture does not provide calcium at the optimum concentration suggested by McCollum, Simmonds, Shipley, and Park (25) when used at the level of 3.7 per cent of the ration. Since September, 1922, we have included 0.8 per cent  $\text{CaCO}_3$  in each 100 gm. of complete ration, thus raising the calcium content from 0.315 to 0.635 per cent. Although the phosphorus content of this ration is approximately 0.67 per cent, including the casein phosphorus, and is, therefore, nearly twice as high as the optimum of 0.36 per cent suggested by McCollum and coworkers, no attempt has been made to reduce this.

*Agar-Agar.*—The agar-agar used has been bacteriological grade, dried in our steam-heated drying drawers until it can be pulverized readily. No purification was attempted.

*Fat.*—Fresh creamery butter, almost without exception a product from the University dairy herd, was melted in an oven at 45–50°C. After several hours standing at this temperature, the curd and water had settled so that the clear supernatant oil could be decanted through heavy filter paper. The filtrate was used as the source of vitamin A. Beginning in September, 1922, the fat in the ration was increased from 5 per cent, furnished by the butter fat, to 15 per cent using Crisco, thereby increasing somewhat the energy value of the ration and giving it a less powdery consistency.

*Dextrin.*—In most cases dextrin has been prepared by autoclaving tapioca at 20 pounds pressure for 4 to 6 hours followed by drying and grinding. In a few experiments the dextrin was made

from corn-starch by autoclaving the starch after moistening with citric acid solution. No further purification of the dextrin was attempted in any of the experiments reported in this paper.

*Vitamin B.*—Commercial wheat embryo with its somewhat variable proportion of bran and endosperm has been used throughout these experiments as a source of vitamin B. The embryo flakes are first subjected to a 12 to 24 hour extraction with ether, the grade "U.S.P. for anesthesia" being employed. This removes the bulk of the oil, following which the ether is evaporated and the dry, essentially oil-free material used for the vitamin B preparation.

We formerly worked with 800 gm. lots of ether-extracted material, but since 1923 when we secured the Lloyd extractor, 5 to 10 kilo lots have been used. In the small lots the type of extractor used provided for siphoning off at intervals the hot alcohol in which the germ was immersed, and replacing this with fresh alcohol. The embryo material was first wet with cold 80 per cent alcohol and thus allowed to siphon over into a 2 liter boiling flask. A second and often a third extraction was made in this way. From this point the extractions were made by hot alcohol coming from the boiling alcohol in the boiling flask. This alcohol was necessarily somewhat stronger, probably about 90 per cent by volume. This extraction was continued so that the embryo was in contact with alcohol for a minimum of 15 hours, but the number of times the alcohol siphoned over varied considerably. The extract was evaporated on a weighed quantity of dextrin and the amount of activated dextrin equivalent to 15 gm. of ether-washed embryo calculated.

Our records show considerable variation in the percentage of substance extracted from commercial wheat embryo by this method. This is no doubt due in part to the variation in bran and endosperm content of the commercial material, and also to the variation in the time and temperature of extraction. 50 extractions of the type described gave an average of 21.9 per cent of the ether-extracted embryo obtained in the alcohol extract. The extremes were 14.2 and 35.6, but 75 per cent fell between 18 and 25 per cent.

The procedure is somewhat different when extracting the vitamin from wheat embryo in large lots in the Lloyd extractor. The ether-washed material is placed in unbleached cotton sacs

holding about 1 kilo which are tied firmly and immersed completely in 95 per cent alcohol in the percolator. A specially located steam jacket on the vaporizing column makes it possible to boil only the upper portion of the alcohol. The condensed alcohol runs into the percolator. The base of the percolator is directly connected with the base of the vaporizing column, so that a constant stream of fresh alcohol is passing through the material in the percolator and the extracted substances are collecting in the vaporizing column. By means of a valve in the line between the percolator and vaporizing column, it is possible to prevent the flow so that the extract can be concentrated in the column. When extracting vitamin B from wheat embryo, this has been done each morning and evening, the extract being drawn off and the fluid replaced by fresh alcohol added to the percolator. Our practice is to make a cold extraction with the strong alcohol until the extract appears to contain very little substance. Finally the alcohol is diluted so that a hot extraction is secured with an 85 to 90 per cent alcohol. The whole process is continued at least 6 days, and sometimes as long as 10 days. The combined extracts are evaporated on dextrin.

The amount of extracted material obtained from wheat embryo in the Lloyd extractor has been somewhat greater than in the small extractor, eleven extractions averaging 24.5 per cent of the ether-extracted embryo. The extremes have been 17.1 and 31.1 per cent, but 75 per cent of the extractions yielded between 20.5 and 28.5 per cent.

The variations in the yield of extract from wheat embryo by alcohol which characterized our procedure should not, in our judgment, be looked upon as a source of error. Although it has meant that the vitamin B content of our rations has probably not been absolutely constant, yet it has been at all times greatly in excess of the minimum requirements as found by others. Steenbock and coworkers (12) have found that 6 per cent of ether-extracted wheat embryo incorporated in the diet is adequate as source of vitamin B for growth of rats on screens. We have at all times used the extracts at a level equivalent to 15 per cent of the diet, which appears to us to provide a large margin of safety. The actual proportion of embryo extract in the diet averaged 3.35 per cent with extremes of 2.13 and 4.7 per cent.

*Laboratory Technique.*—From the start our rats have been kept in colonies of five to seven animals in cages with approximately  $50 \times 50$  cm. floor space. All rats have been of our own breeding, both inbred and cross-bred rats being used. They have been in part albino and in part black and white stock, both strains attaining adult size at about 20 weeks of age for the females and about 24 weeks for the males. The average adult weight is 200 gm. for the female and 325 to 350 gm. for the males. All rats are placed on experiment when 4 to 5 weeks of age, at which time they weigh 50 to 70 gm. Our stock diet is a uniform standard mixture of whole cereals, legume seeds, alfalfa leaves, dried meat, cod liver oil, common salt, and sufficient calcium carbonate to bring the percentage of calcium to 0.65 per cent. This diet and fresh, raw, liquid milk daily are before our breeding stock at all times. Lettuce or carrots are given at intervals.

The experimental ration is kept before the experimental animals so that consumption is *ad libitum*. The feeding cup provides for a fairly accurate record of food intake. Distilled water containing a trace of iodine is provided. The cages are thoroughly cleaned each week. Body weight and food intake records are made at weekly intervals.

Beginning with the second experiment of this series in July, 1922, the rats were kept on a screen floor  $\frac{1}{2}$  to  $\frac{3}{4}$  inches above the floor of the cage which was kept covered with sawdust. Our object was to prevent consumption of the bedding which we regarded as a possible factor in supplying unknown substances in the diet. For the 1st year the screen used was a 4 mesh per inch galvanized wire, then a 3 mesh wire was substituted. Steenbock and coworkers (12), Dutcher and Francis (26), Heller, McElroy, and Garlock (27), and Smith, Cogwill, and Croll (28) have since emphasized the importance of coprophagy in nutrition experiments with rats, particularly in increasing substantially the vitamin B requirements for growth when feces consumption is reduced.

Our own extensive experiments substantiate this conclusion, but we are convinced also that the results obtained by preventing as far as possible access to the excreta are not to be explained solely on the grounds of vitamin B. This is brought out clearly in the present series of experiments and will be shown most definitely in the subsequent papers in this series.

*Results.*

Charts 1 and 2 show the growth curves of sixteen lots, totalling 88 rats, fed the presumably complete basal ration described. It is seen that some tests have been made in each of 5 successive years. Lot 1 is the original experiment with animals on sawdust. In all subsequent experiments coprophagy was eliminated as far as possible by the use of screens as described under "Technique."

With the exception of Lot 1, on sawdust, uniform failure of the animals to grow normally characterized the experiments. In Lot 1 growth was good during the 5 months the experiment lasted, approaching the older Donaldson average for both males and females. On the subsequent experiments a few animals attained this average for a few weeks, but in all cases except Lot 15, which was taken off at the end of 11 weeks while still slowly growing, there was eventually maintenance only and then decline.

The progress of the deficiency disease was characterized by partial but not severe anorexia, rough, often excessively greasy fur, and generally poor condition. As seen on the charts, the food intake ranged from 30 to 46 gm. (135 to 210 calories, calculated) per rat per week which cannot be regarded as excessively low; in fact it should be considered normal for some of the lots as judged from the size of the rats. There was never any indication of xerophthalmia, polyneuritis, or rickets. In spite of the failure of appetite and poor appearance the animals in most cases were lively and playful.

Certain special features of the technique or results in the individual lots are mentioned below.

*Lot 1.*—This was the only group maintained on sawdust. Each of the three females produced one litter at a normal age for the first litter. Females 227 and 228 ate their young. Female 227 declined and died within 2 weeks after parturition. Female 229 nursed its litter of five for 21 days, but at the end of this time, they weighed only 9 gm. each and were very weak. The average food consumption per rat per week was 60 gm. in this group.

*Lot 2.*—These rats were on a 4 mesh screen elevated 1 inch above the floor of the cage. Female 863 was found pregnant on autopsy. There was a slight lung infection in each of the rats in this lot. The mean weekly food consumption for each rat was 36.6 gm.

*Lot 3.*—Beginning with this lot the fat content of the ration was increased to 15 per cent and consisted of 5 per cent butter fat and 10 per cent Crisco.

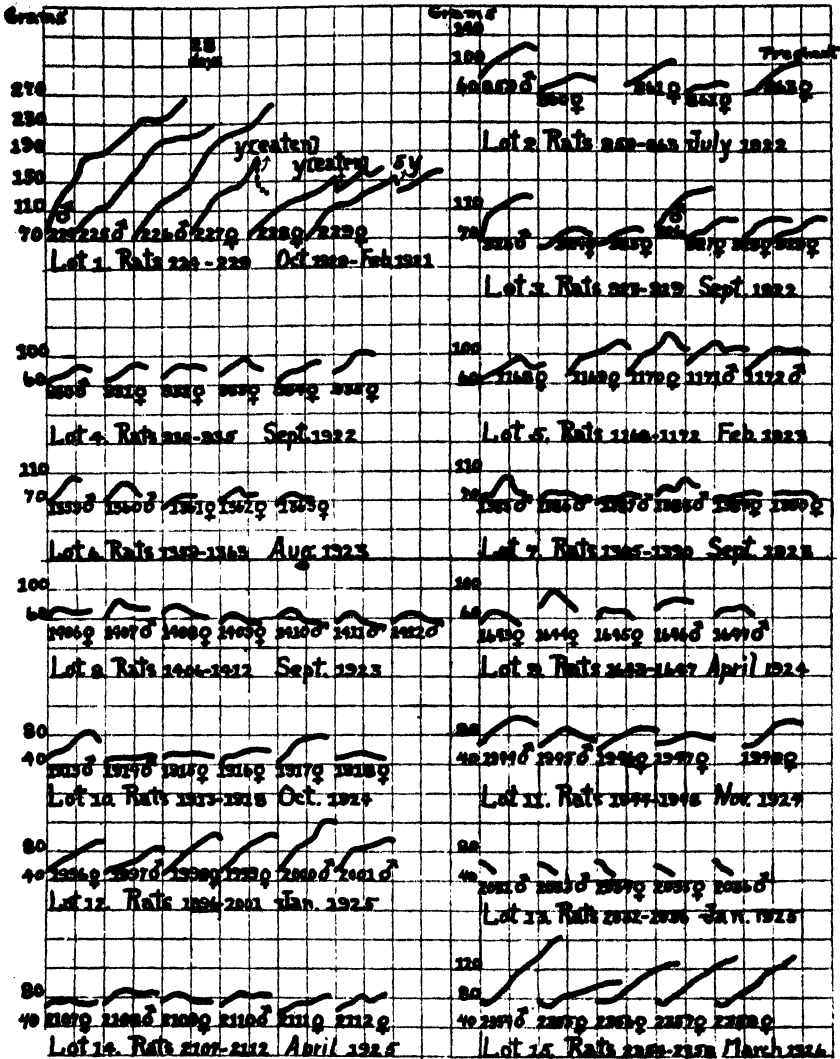


CHART 1. This chart shows the growth curve of fifteen lots of rats fed a presumably complete synthetic diet of the following composition: highly purified casein 18 parts, filtered butter fat = 5 or 15 parts, Crisco = 0 or 10 parts, McCollum Salt Mixture 185 = 3.7 parts, agar-agar = 2.0 parts, calcium carbonate = 0.8 parts, wheat embryo extract  $\approx$  15 parts of ether-extracted embryo, tapioca dextrin to 100 parts. The chart shows the sex of the animals and the date of each experiment. Lot 1 was kept on sawdust and the remainder on screens. Lot 13 was self-fed the ingredients of the ration instead of the mixture. The average weekly food intake per rat in the various lots is as follows: Lot 1, 60 gm.; Lot 2, 36.6; Lot 3, 45.6; Lot 4, 44.1; Lot 5, 48.9 for the first 5 weeks and 26.6 for the last 4 weeks; Lot 6, 36; Lot 7, 30; Lot 8, 45; Lot 9, 36; Lot 10, 38; Lot 11, 41; Lot 12, 30; Lot 13, 34; Lot 14, 30; Lot 15, 43.

The weekly food intake per rat of this lot should have been sufficient for normal growth as it averaged 45.6 gm.

*Lot 4.*—Beginning with this lot the calcium content of the basal ration was increased to 0.65 per cent by including 0.8 per cent  $\text{CaCO}_3$  in the ration. The weekly food intake of this lot which averaged 44.1 gm. per rat was in excess of the energy needs per unit of body weight, but the animals were very thin and had very rough fur at the close of the experiment.

*Lot 5.*—The food intake of this lot was more than adequate for the first 5 weeks of the experiment, averaging 48.9 gm. per rat per week, but this fell to 26.6 gm. during the last 4 weeks.

*Lot 6.*—Beginning with this lot the floor of the cages was changed from a 4 mesh to a 3 mesh screen. The food intake averaged 35 gm. per rat per week.

*Lot 7.*—Beginning with this lot the case in purification and wheat embryo extractions were made with the Lloyd extractor. These animals were characterized by their poor condition and very greasy fur. Corn-starch dextrin was used in this experiment. The food intake was low, averaging only 30 gm. per rat per week.

*Lot 8.*—This experiment had certain characteristics strikingly different from any which preceded it. The animals finished the experiment in good condition and with smooth, normal fur. Although the food intake was ample, averaging 45 gm. per rat per week, there was no growth whatever.

*Lot 9.*—There were no special features. The rats were in poor condition at the close of the experiment. The food intake was subnormal, averaging only 36 gm. per rat per week.

*Lot 10.*—The food intake of 38 gm. per rat per week was too great to account for the complete failure of four of the six rats to grow. The rats were very thin and their fur very greasy.

*Lot 11.*—In this experiment the basal ration, except the butter fat, was mixed with water and cooked in a double boiler for about 20 minutes. The gelatinized mass was dried, ground, and mixed with 5 per cent butter fat before feeding. The food intake was fair, averaging 41 gm. per rat per week, and should have given much better growth than resulted. The rats had very greasy fur in this experiment.

*Lot 12.*—The ration of this lot contained no Crisco, this being replaced by butter fat. The growth failure in this test is obviously due in part to the low food intake of only 30 gm. per rat per week. The fur became excessively greasy in this experiment.

*Lot 13.*—This was an interesting modification of the usual procedure. A self-feeding device was constructed so that the rats could partake of each ingredient of the basal ration *ad libitum*. The casein was moistened to a granular mass. The food consumption of the five rats during the 2 weeks of the experiment and the composition of the diet which the consumption represented are shown in Table I. The intake of 34 gm. per rat per week in this experiment shows that the decline in weight was not accounted for by too low an energy intake. The vitamin A intake, as butter fat, was adequate on a proportionate basis, but that of the vitamin



B preparation was only little over one-half of that represented by the usual basal ration which contains 10 or 11 per cent of embryo preparation. It is not possible to state definitely that 6.5 per cent of vitamin B preparation is too low since it represents about 8 per cent of extracted wheat embryo. The growth failure seems, therefore, to be attributable chiefly to the low intake of salt mixture and highly purified protein.

The relative high intake of agar is one of the surprising features of the results.

*Lot 14.*—The ration was like that of Lot 12, containing no Crisco. The fat content, however, was only 5 per cent, this being butter fat. The low food intake of only 30 gm. per rat per week was no doubt a contributing cause of growth failure. It seems evident, however, that the food intake should have stimulated some growth instead of merely maintaining the animals. This idea is strengthened by comparing the growth of this lot with that of Lot 12, which had the same food intake.

TABLE I.

*Food Intake and Composition of Diet Eaten by Five Rats Allowed Self-Choice of Ingredients of Purified Synthetic Diet.*

	Food intake.	Proportion of total.
	gm.	per cent
Casein.....	17	5.03
Salts.....	3	0.89
Dextrin.....	240	71.00
Butter fat.....	18	5.32
Crisco.....	16	4.73
Wheat embryo extract (on dextrin).....	22	6.51
Agar.....	22	6.51
2 weeks total.....	338	99.99

*Lot 15.*—The ration for this lot was the usual Ration 5 containing 5 per cent butter fat and 10 per cent Crisco. The wheat embryo extract was prepared from a commercial embryo containing a low percentage of bran and the alcoholic extraction was continued for 2 weeks. The percentage of extract secured was somewhat higher than the average; namely, about 31 per cent. While growth was by no means normal in this lot, it was considerably better than a large majority of the lots. Although the food intake was moderately good, averaging 43 gm. per rat per week, it could not have been the sole cause of the better growth because Lots 3, 4, and 8 with better food intake made much poorer growth. The condition of the animals at the end of the 11 weeks was not nearly as good as the growth curves indicate. The fur was thin and somewhat greasy. Certain parts of the back and flanks were almost bald, and the animals had an under-nourished appearance.

Chart 2 shows the behavior of three mature breeding rats from the stock colony when placed on screens and given Ration 5. The females had borne and reared litters. They became pregnant a few days after going on the experiment, and each gave birth to one litter, which was devoured within a day or 2. After the first few weeks, all three animals declined in weight and became very miserable in appearance. Their coats were thin and shaggy.

Evidently the animals were able to draw on body stores for a limited time to supply the missing factors in Ration 5. That the male rat maintained its weight successfully during the gestation period of its mates, indicates that the successful termination of the pregnancies was due to some storage in the animal body common

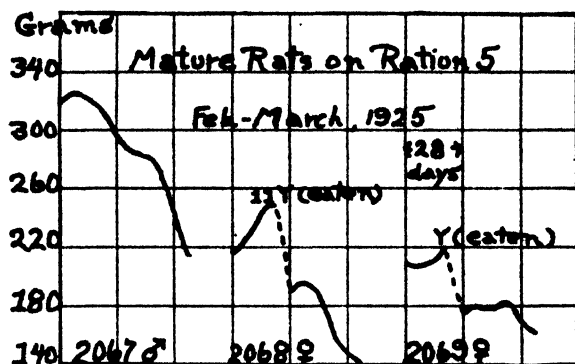


CHART 2.

to each of the rats and that this reserve was depleted about the same time for each of them. Very clearly Ration 5 is not only inadequate for growth as shown in Chart 1, but also inadequate for the maintenance of mature animals.

#### DISCUSSION.

We have described in this paper a sufficient number of experiments to show conclusively that a diet may be constructed which is adequate both qualitatively and quantitatively for growth according to generally accepted views, but which fails completely to support either growth of young rats or maintenance of mature ones except for a few weeks.

This result appears, from the evidence presented, to have been

brought about by one or both of two procedures: (1) the use of more highly purified protein, and (2) the suppression as far as possible of coprophagy by means of screen floors in housing the rats. These apparently simple modifications of procedure produce such a profound effect that it seems reasonable to suppose that they involve some fundamental requirement for growth. From the first failure encountered in the case of Lot 2 we have recognized the possibility that this may be due either to a substance or substances, the necessity of which is already recognized, but which are supplied in insufficient amounts in our basal diet, or to some other necessary growth factor, as yet recognized only vaguely, if at all, the need for which is emphasized by our procedure.

The current explanation of growth failure of rats kept on screens is that of an augmented vitamin B requirement. The possibility of this being the cause of our results must, however, be viewed in the light of the vitamin B content of the basal diet. As explained in a previous paragraph, the extract of commercial wheat embryo supplied in the basal diet is relatively so large in comparison with the requirements as judged by the experience of others (12, 29), as well as numerous experiments of our own, using other sources of vitamin B, which will be reported in a later paper, that we have been forced to the opinion that the deficiency revealed by the use of screens in our experiments is not solely, if at all, one of vitamin B as this factor is ordinarily conceived.

We have also considered and studied in some detail the possibility that the failure of the rats to grow normally is to be attributed to a deficiency of vitamin A, vitamin D, vitamin C, or bios. In addition we have made comparisons of various salt mixtures. The protein factor has been given special study inasmuch as we regard the purification of casein as a special feature of our technique. We have found that the purification of the protein is an important factor. All of these studies will be reported in a subsequent paper. It may be said, however, that we have not been able to explain the subnormal growth entirely on the basis of a deficiency of recognized nutrients. On the other hand, we have noted variations in growth-promoting qualities of vitamin A and vitamin B preparations which cannot be explained on the grounds of a deficiency of these vitamins. We are persuaded that the basic deficiency or deficiencies can be both brought out and augmented

by the biological changes in the digestive tract caused by the suppression of coprophagy. Our studies of this phase of the problem will also be reported separately.

The possibility of our results being related to those of Emmett and Luros (15, 16) requires consideration. Certain of the data to be presented in a later paper suggest such a relationship. At any rate we found in certain experiments that U.S.P. lactose benefited our animals greatly; however, the fact that the lactose was still beneficial after several recrystallizations from 70 per cent alcohol is less suggestive of a vitamin factor than of some other cause. On the other hand, the fact that different lots of lactose fail to be uniformly beneficial tends to support the idea of a labile vitamin.

More recently, we have had occasion to consider the relation of our results to the suggestion of Smith and Hendrick (19) and Goldberger and coworkers (20) that rats require a growth factor retained by autoclaved yeast. We have found indications that autoclaved yeast stimulates animals to greater growth on our basal diet. It seems improbable, however, that the heat-labile factor suggested by Emmett and Luros (15, 16) is identical with the heat-stable factor in yeast. The situation with respect to our results is, therefore, complicated rather than clarified by these considerations. Whether one considers that our results support the existence of one or both of the factors suggested, or whether another or other vitamin-like substances is indicated, the data presented in this paper show conclusively that the requirements for normal growth of rats are not satisfied by a balanced diet made up of generally recognized nutrients when the technique involves a more rigid purification of the protein and a more rigid laboratory procedure in housing the animals.

We are engaged in organizing the results of our further study of this problem. These we believe will show in a more definite manner than the more or less negative evidence presented in this paper, the existence of essential growth factors not at present recognized.

#### SUMMARY AND CONCLUSIONS.

It has been shown by experiments repeated at intervals covering nearly 5 years that young rats fail to grow normally if at all when fed a basal diet of specially purified casein, dextrin, wheat embryo

extract, butter fat, mineral salts, and agar, in proportions presumably adequate for satisfying the growth requirements for protein, energy, mineral elements, and vitamins A and B, and housed so as to repress the natural coprophagistic habits of the species.

We have concluded from these data that the requirements for normal growth of the rat involve other nutrients than those usually recognized and incorporated in our basal diet. Further studies referred to but not presented in this paper, suggest a need for vitamin-like factors other than those at present generally recognized.

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## THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

### V. ON THE CHEMICAL NATURE OF THE ALDOBIONIC ACID FROM THE SPECIFIC POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS.

BY MICHAEL HEIDELBERGER AND WALTHER F. GOEBEL.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

(Received for publication, June 25, 1927.)

The so called soluble specific substances of the three fixed types of *Pneumococcus* appear identical with three chemically distinct, serologically type specific polysaccharides produced by these organisms during growth in culture media (1). The immunological significance and the chemical nature of these bacterial carbohydrates have been discussed in detail in earlier publications from this laboratory. The present communication deals with the chemical nature of an aldobionic acid, the fundamental building stone of the polysaccharide derived from Type III pneumococcus.

This aldobionic acid, the product of hydrolysis of the Type III specific carbohydrate, has been shown (2) to have the formula  $C_{11}H_{19}O_{10}COOH$  and to be built up from a hexose (glucose) and a hexose-uronic acid of unknown nature, in such a manner that the carboxyl group and one aldehydic group remain free. It is of interest to extend the investigation of this substance, for it not only appears unique in the field of sugar chemistry, but important in that it or its isomers are found among the hydrolytic products of specific carbohydrates from other microorganisms. The present report aims to identify the hexose-uronic acid which forms half of the molecule of the aldobionic acid, and to explain the nature of the glucosidic linkage which binds the sugar to the acid.



**EXPERIMENTAL.***1. Preparation of the Aldobionic Acid.*

30 gm. of air-dry specific polysaccharide (prepared as in Paper IV (2)) were dissolved in 120 cc. of 75 per cent sulfuric acid (by weight) at 0°. After standing overnight in the ice box the solution was diluted to 3 liters and boiled 5 hours under reflux. The sulfuric acid was then quantitatively removed with highly purified barium hydroxide and the barium sulfate washed free from reducing sugars. The combined filtrates were concentrated to 200 cc. *in vacuo*, boiled with a little norit and an excess of calcium carbonate, filtered, and the filtrate concentrated to 100 cc. *in vacuo*. The solution, which contained small amounts of glucose and the calcium salt of the aldobionic acid, was poured into 10 volumes of methyl alcohol. In this manner the crude calcium aldobionate was freed from reducing sugars. The suspension was filtered and washed two or three times with methyl alcohol. 24 gm. of crude calcium aldobionate were thus obtained.

*2. Preparation of Pure Calcium Aldobionate.*

The crude salt was dissolved in twice its weight of water, and to the solution was added alcohol in small portions. After each addition of alcohol the mixture was centrifuged. Enough alcohol was added so that after the final centrifugation the supernatant liquid, still containing a large part of the original calcium salt, remained as a pale straw-colored solution. This was decanted from the deeply colored lower oily layer and saved. The lower layer was again dissolved in an equal volume of water, and treated as before with alcohol, the straw-colored supernatant liquid again being saved. After fractionating the lower layer two or three times more, a deeply colored oil was obtained which was discarded, and the combined supernatant liquids were concentrated *in vacuo* and poured into 10 volumes of methyl alcohol. 18 gm. of purified calcium aldobionate were finally isolated. The substance gave the following analysis:

0.1028 gm. substance: 0.1438 gm. CO<sub>2</sub> and 0.0490 gm. H<sub>2</sub>O.

0.1096 " " : 0.0092 " CaO.

Calculated for (C<sub>12</sub>H<sub>19</sub>O<sub>12</sub>)<sub>2</sub>Ca. C 38.33 per cent, H 5.07 per cent, Ca 5.33 per cent. Found. C 38.14 per cent, H 5.33 per cent, Ca 6.00 per cent.

The free aldobionic acid was obtained by adding a little less than the calculated amount of oxalic acid to a 5 per cent solution of the calcium salt, filtering off the calcium oxalate, concentrating the filtrate to dryness *in vacuo*, and dissolving the residue in methyl alcohol. The alcoholic solution of the aldobionic acid was filtered from the small amount of insoluble calcium aldobionate, and the filtrate was evaporated to dryness *in vacuo*. The sugar, if properly manipulated, will puff up into a spongy mass, and when completely dry it may be readily broken up and scraped from the flask.

### 3. Oxidation of the Aldobionic Acid with Barium Hypoiodite.

5.0 gm. of aldobionic acid were dissolved in a small amount of water and the solution was oxidized with barium hypoiodite by a method previously described (3). The solution of the oxidation product, after being freed completely from inorganic constituents, was boiled with calcium carbonate and a small amount of norit. It was then filtered and concentrated to 30 cc. *in vacuo*. By the gradual addition of alcohol a precipitate of the calcium salt of the oxidized aldobionic acid, which will be termed glucuronogluconic acid, separated out. This calcium salt is far less soluble than is that of the aldobionic acid, 25 cc. of alcohol sufficing to remove it from solution. The calcium salt was filtered off and weighed. 5.2 gm. were recovered.

0.1000 gm. substance: 0.1284 gm.  $\text{CO}_2$  and 0.0426 gm.  $\text{H}_2\text{O}$ .

0.1368 " " : 0.0188 "  $\text{CaO}$ .

Calculated for  $\text{C}_{10}\text{H}_{18}\text{O}_9(\text{COO})_2\text{Ca}$ . C 35.12 per cent, H 4.42 per cent, Ca 9.76 per cent. Found. C 35.01 per cent, H 4.77 per cent, Ca 9.82 per cent.

### 4. Properties of the Glucuronogluconic Acid.

This dicarboxylated acid, when free from calcium, forms a white amorphous powder soluble in methyl alcohol, and less soluble in ethyl alcohol. It is soluble in hot glacial acetic acid, and, on cooling, droplets of oil separate which do not crystallize on standing. The free carboxylated sugar is a strong acid, and an aqueous solution colors Congo red paper a vivid blue. The calcium salt shows  $[\alpha]_D = -7.5^\circ$ .

The acid itself gives a strong naphthoresorcinol test and on distillation with 12 per cent hydrochloric acid it yields 17 per cent

of furfural. The acid is non-reducing, but when boiled with strong hydrochloric acid reducing sugars appear. On hydrolyzing for 15 hours with normal hydrochloric acid it yields a maximum of 24.8 per cent reducing sugars (calculated as glucose). It was thought possible to hydrolyze the acid with sodium amalgam as Levene and La Forge (4) hydrolyzed chondrosin, but experiments in this direction failed. The acid is extremely stable to hydrolysis, both by acids and alkalies.

5. *Hydrolysis of the Aldobionic Acid with Bromine and Hydrobromic Acid.*

2.0 gm. of aldobionic acid were dissolved in 50 cc. of *N* hydrobromic acid and to the solution was added 0.5 cc. of bromine. The mixture was boiled under a reflux for 20 hours. It was necessary to replace the bromine from time to time. At the end of the hydrolysis the solution gave only a very faint naphthoresorcinol test and showed no reduction. The solution was then evaporated *in vacuo* to remove hydrobromic acid and bromine. The remaining traces of acid were removed with silver sulfate and the silver bromide was filtered off. The silver ion in the filtrate was removed with hydrogen sulfide and after filtration the sulfate ion was removed quantitatively with barium hydroxide. A filtrate free from inorganic constituents was thus obtained. This filtrate was evaporated to 2 cc. *in vacuo*, made strongly alkaline with 50 per cent potassium hydroxide, and then acidified with glacial acetic acid. The solution was seeded with a small crystal of potassium acid saccharate and was placed in the ice box. After standing 24 hours crystals of potassium acid saccharate (0.20 gm.) were filtered from the mother liquor. The crude salt was recrystallized from 1 cc. of boiling water.

0.0474 gm. substance gave 0.0168 gm.  $K_2SO_4$ .

Calculated for  $COOK(CHOH)_4COOH$ , K 15.75 per cent.

Found.

“ 15.89 “ “

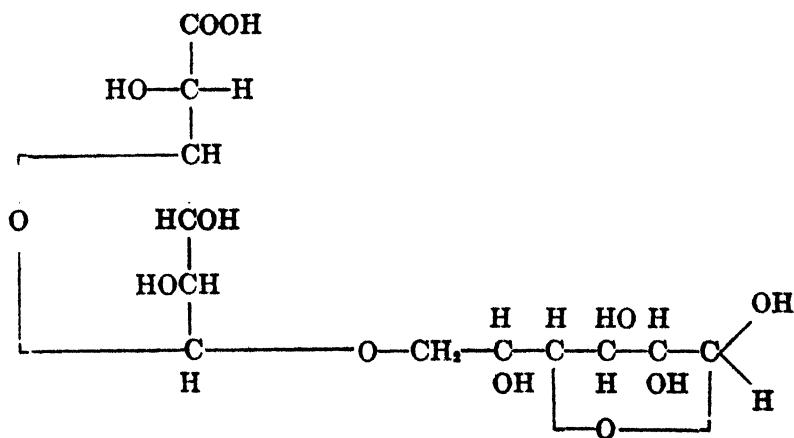
On substituting glucose for the aldobionic acid, a repetition of the above experiment gave no potassium acid saccharate.

DISCUSSION.

Since it had previously been shown that the aldobionic acid, the chief hydrolytic product of the specific polysaccharide of Type

III pneumococcus, is composed of glucose and a hexose-uronic acid of unknown nature, united in such a manner that one aldehydic group remains reactive in the bionic acid molecule, it must necessarily be assumed that the sugar and sugar acid are combined in glucosidic linkage either through the aldehydic group of the glucose or through that of the hexose-uronic acid. If the linkage were of the first type, then the product obtained by oxidation of the free aldehydic group with barium hypoiodite would obviously be a true glucoside of a dibasic hexose acid; if the linkage were through the aldehydic group of the hexose-uronic acid, the latter would be intact after oxidation, whereas the free aldehydic group of the glucose would be oxidized. Since the glucuronogluconic acid, obtained by the oxidation of the aldobionic acid, still gives a naphthoresorcinol test and still yields the same amount of furfural on distillation as it did before oxidation, one must assume that the hexose-uronic acid does remain intact and that the free aldehydic group of the aldobionic acid is actually the reducing group of the glucose half of the molecule and not that of the uronic acid. It has been shown above, that the aldobionic acid yields saccharic acid on hydrolysis in the presence of bromine. Since glucose does not yield this acid under these conditions, one must necessarily assume that the saccharic acid formed in the above experiment owes its origin to the hexose-uronic acid part of the aldobionic acid molecule and that the hexose-uronic acid is therefore glucuronic acid.

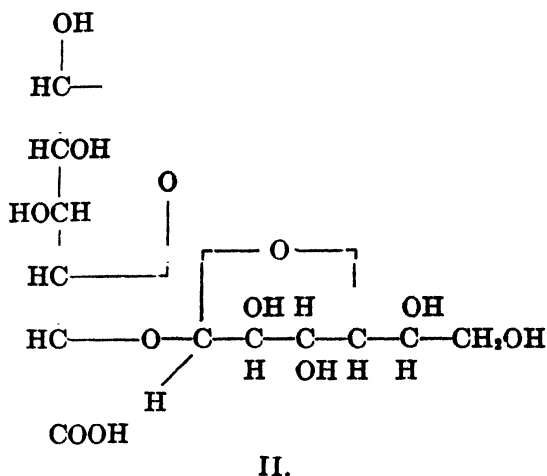
The formula (I)



I.

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is in accord with the results of the experiments which have been performed, whereas the isomeric formula (II)



is not.

Whether the linkage between glucuronic acid and glucose is through carbon atom (6), in formula (I), or through one of the other carbon atoms, remains to be determined.

### SUMMARY.

The aldobionic acid  $\text{C}_{11}\text{H}_{19}\text{O}_{10}\text{COOH}$  isolated from the hydrolytic products of the specific polysaccharide of Type III pneumococcus has been shown to be a compound of glucuronic acid and glucose, combined in glucosidic linkage through the aldehydic group of glucuronic acid and one of the carbon atoms of glucose.

In conclusion the writers wish to express their gratitude to Dr. P. A. Levene for his many helpful suggestions, particularly his suggestions on the manipulation of sugar acids.

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## THE SOLUBLE SPECIFIC SUBSTANCE OF FRIEDLÄNDER'S BACILLUS.

### IV. ON THE NATURE OF THE HYDROLYTIC PRODUCTS OF THE SPECIFIC CARBOHYDRATE FROM TYPE A FRIEDLÄNDER BACILLUS.

By WALTHER F. GOEBEL. .

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

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The methods of isolation and the immunological significance of specific carbohydrates from the three fixed types of *Pneumococcus* and of Friedländer's bacillus have been described in a series of communications from this laboratory (1). These unusual polysaccharides, which are believed to be identical with the capsular material of the microorganisms from which they are derived, have been isolated as nitrogen-free,<sup>1</sup> amorphous compounds possessing marked acidic properties. They are complex carbohydrates built up apparently from molecules of hexose and hexuronic acids in varying proportions. Although no contention has been made that these polysaccharides are pure distinct chemical individuals, a fair amount of evidence has been gathered which supports this view.

These carbohydrates have many physical and chemical characteristics in common; they show distinct differences, however, in the degree to which they rotate the plane of polarized light, in their acid equivalent values, and in the selective specificity which they show toward antibacterial serum.

The properties of these carbohydrates are briefly given in Table I.

The polysaccharide from the Type A Friedländer bacillus resembles the *Pneumococcus* Type III specific carbohydrate in some of its chemical properties, though immunologically the two compounds are totally unrelated. Both polysaccharides have

<sup>1</sup> With one exception.

low acid equivalent values and both give a strong naphthoresorcinol test, a test which indicates the presence of glucuronic acid or an isomer within the molecule. A sugar acid, termed aldobionic acid, has been isolated from the hydrolytic products of

TABLE I.

*Soluble Specific Substances of the Three Fixed Types of Pneumococcus and of Friedländer's Bacillus.*

	$[\alpha]_D$	Acid equivalent.	C	H	N	Ash.	Reducing sugars on hydrolysis (calculated as glucose).	Sugars liberated on hydrolysis.	Highest dilution giving precipitate with immune serum.
							per cent		
Pneumococcus type. I	+300°		43.3	5.8	5.0	0.0	28	(Amino sugar) (galacturonic acid).*	1:6,000,000
II	+74°	1250	45.8	6.4	0.0	0.0	70	Glucose (aldobionic acid).	1:5,000,000
III	-33°	340	42.6	5.6	0.0	0.0	75	Glucose, aldobionic acid.	1:6,000,000
Friedländer type. A	-100°	430	43.95	6.0	0.0	0.0	65	Glucose, aldobionic acid.	1:2,000,000†
B	+100°	680	44.6	6.1	0.0	0.0	70	" "	1:2,000,000
C	+100°	680		0.0	0.0	0.0	70	" "	1:2,000,000

\* Compounds given in parentheses have not been completely identified, but evidence of their presence has been obtained.

† Rabbit serum.

the *Pneumococcus* Type III polysaccharide (2). This acid appears to be built up from 1 molecule of glucuronic acid and 1 molecule of glucose. The Friedländer Type A specific carbohydrate is in itself a strong acid and since it gives a characteristic

test for glucuronic acid, it was thought that the hydrolytic products of the Friedländer carbohydrate might contain an aldobionic acid either identical or isomeric with the aldobionic acid of the *Pneumococcus* Type III soluble specific substance.

An investigation into the nature of the hydrolytic products of the Friedländer Type A specific carbohydrate was therefore undertaken with the hope of isolating this acid and of throwing light on the nature of the polysaccharide molecule as a whole.

#### EXPERIMENTAL.

##### *Hydrolysis of the Specific Polysaccharide.*

34 gm. of dry specific polysaccharide prepared from cultures of the A strain of Friedländer bacillus by a method previously described (1, b) were dissolved in 1 liter of normal sulfuric acid and the solution was boiled for 5 hours under a reflux. The sulfuric acid was quantitatively removed with barium hydroxide, and the solution was filtered from the barium sulfate. The clear yellow filtrate was boiled with a little norit and an excess of calcium carbonate and was filtered. This filtrate, after concentration to 50 cc. *in vacuo*, was poured into 10 volumes of methyl alcohol. The precipitate, the calcium salts of sugar acids, was filtered from the alcoholic solution of true sugars. The alcoholic filtrate was evaporated to dryness *in vacuo*. 13.0 gm. of dry calcium salt and 19.0 gm. of sugars were recovered.

##### *A. Properties and Identification of Components of the Sugar Acid Fraction.*

###### *1. Purification of the Calcium Aldobionate and Properties of the Aldobionic Acid.*

The calcium salt, a yellow amorphous powder, was dissolved in  $1\frac{1}{2}$  times its weight of water and was precipitated with an equal volume of absolute alcohol. The mixture was centrifuged. A pale yellow supernatant fluid was separated by decantation from a deep yellow oily lower layer. The latter was redissolved in an equal volume of water and was reprecipitated with  $1\frac{1}{2}$  volumes of alcohol. The suspension was centrifuged and the supernatant liquid was added to the supernatant liquid from the first purifica-



tion. The deep yellow lower layer was discarded. The combined supernatant liquids from the purification were evaporated to a syrup *in vacuo* and were finally poured into methyl alcohol. 9.2 gm. of dry substance were recovered.

The salt was next dissolved in water and was treated with slightly less than the theoretical amount of oxalic acid. The calcium oxalate was filtered off, and the filtrate was evaporated to dryness *in vacuo*. The free sugar was dissolved in methyl alcohol and was separated from a slight amount of insoluble calcium salt by centrifugation. The methyl alcoholic solution of the sugar acid was evaporated to dryness *in vacuo*, and the residue was dissolved in 100 cc. of water. A pale yellow solution was obtained. This solution was chilled to 0° and to it were added 10 cc. of 25 per cent basic lead acetate solution. A deep yellow precipitate was formed which was removed by centrifugation. The precipitate was discarded. The colorless supernatant liquid was treated with basic lead acetate solution until no further precipitation resulted. The precipitate was separated by centrifugation. The lead salt of the sugar acid was next suspended in water, and was treated with hydrogen sulfide. After filtering off the lead sulfide, the free sugar acid was obtained from the filtrate by evaporation to dryness *in vacuo*. The free sugar acid, as obtained in this manner, gave a strong naphthoresorcinol test and reduced Fehling's solution vigorously. 0.3124 gm. when dissolved in 15 cc. of water showed an optical rotation of  $-2.25^\circ$  in a 2 dm. tube. This is equivalent to  $[\alpha]_D = -54^\circ$ . 0.1000 gm. of substance neutralized 3.95 cc. of N/14 sodium hydroxide, an acid equivalent of 354. Its reducing power as measured both by the Shaffer-Hartmann (3) and by the Willstätter-Schudel (4) method, is just 50 per cent that of glucose.

The substance is apparently similar to the aldobionic acid obtained from the hydrolysis of the *Pneumococcus* Type III specific polysaccharide and seems to be built up from two sugars, a hexose and a hexose-uronic acid, in such a way that the carboxyl and one aldehyde group remain free in the molecule.

0.1013 gm. substance: 0.1496 gm. CO<sub>2</sub> and 0.0492 gm. H<sub>2</sub>O.

Calculated for C<sub>11</sub>H<sub>19</sub>O<sub>10</sub>COOH. C 40.45 per cent, H 5.66 per cent.

Found.

" 40.31 " " " 5.40 " "

## 2. Identification of Components of the Aldobionic Acid.

(a) *Identification of the Sugar Half of the Molecule.*—1.0 gm. of aldobionic acid was dissolved in 50 cc. of N sulfuric acid and the solution was boiled for 15 hours under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide. The filtrate was boiled with calcium carbonate, filtered, and the solution was evaporated to dryness *in vacuo*. The residue was shaken with methyl alcohol and again filtered. In this manner the alcohol-insoluble calcium salt of unhydrolyzed sugar acid was separated from the hexose liberated by hydrolysis. The free sugar, of course, was in the alcoholic filtrate. This alcoholic solution was evaporated *in vacuo* to dryness. The residue was taken up in water and was diluted to 50 cc. In a 2 dm. tube the solution gave a rotation of  $+0.57^\circ$ , or  $[\alpha]_D = +47.5^\circ$ . An analysis by the Shaffer-Hartmann method showed the solution to contain 0.30 gm., calculated as glucose. The remaining solution was treated with 3.5 mols of phenylhydrazine acetate and was heated 1 hour on the water bath. The crystalline osazone which formed was filtered off and washed with a few drops of methyl alcohol. The yield was 0.16 gm. The product melted at  $203-204^\circ$ . Its initial  $[\alpha]_D$  was  $-57.2^\circ$ , mutarotating to  $-24^\circ$  after 48 hours.

From the melting point of the osazone, its direction of mutarotation, and finally from the specific rotation of the sugar solution itself, it is justifiable to conclude that this product of the hydrolysis of the aldobionic acid is glucose, and that the hexose half of the molecule is therefore glucose.

(b) *Identification of the Sugar Acid Half of the Molecule.*—2.0 gm. of aldobionic acid were boiled under a reflux with 50 cc. of N hydrobromic acid and 0.5 cc. of bromine. The bromine was replaced from time to time. At the end of 15 hours the hydrobromic acid and bromine were removed as completely as possible by distillation *in vacuo*. The remaining traces of hydrobromic acid were removed with silver sulfate, the excess silver ion was removed with hydrogen sulfide, and, after filtration, the sulfate ion was removed quantitatively with barium hydroxide. The aqueous residue, containing no inorganic constituents, was evaporated to 2 cc., and was then made alkaline with 50 per cent potassium

hydroxide. After acidification with glacial acetic acid, crystals of potassium acid saccharate separated from the solution. 0.2 gm. was recovered. After recrystallization from water the substance had the following analysis.

0.0500 gm. substance: 0.0176 gm.  $K_2SO_4$ .

Calculated for  $HOOC(CHOH)_4COOK$ . K 15.75 per cent.

Found.

" 15.78 " "

It has been shown that the hexose half of this aldobionic acid molecule is glucose. Glucose does not yield saccharic acid under the conditions of the above experiment. One must therefore assume that the saccharic acid owes its origin to the hexose-uronic acid half of the aldobionic acid molecule, and that the hexose-uronic acid is therefore glucuronic acid.

(c) *Oxidation of the Aldobionic Acid with Barium Hypiodite.*—0.6 gm. of aldobionic acid was oxidized to the dibasic sugar acid by means of barium hypiodite (5). The glucuronogluconic acid was isolated as its calcium salt.

0.0861 gm. substance: 0.1096 gm.  $CO_2$ , 0.0358 gm.  $H_2O$ .

Calculated for  $C_{10}H_{18}O_9(COO)_2Ca$ . C 35.12 per cent, H 4.42 per cent.

Found.

" 34.71 " " " 4.65 " "

When analyzed by the method of Pervier and Gortner (6), the substance yielded 15 per cent of furfural. Since glucuronic acid yields about one-third of the amount of furfural liberated by pentoses under corresponding treatment, this figure would correspond roughly to 50 per cent of glucuronic acid within the molecule. The saccharobionic acid, when isolated as its calcium salt, is a non-reducing, water-soluble, amorphous compound which gives a strong naphthoresorcinol test.

#### *B. Properties and Identification of Components of the Sugar Fraction.*

The so called sugar or alcohol-soluble fraction which composed approximately two-thirds of the total hydrolytic products of the Type A specific polysaccharide was an optically inactive amorphous substance having a reduction value equal to 75 per cent that of glucose. The material gave only a faint naphthoresorcinol test; it gave no orcinol test.

### 1. Identification of Glucose.

1.0 gm. of substance was dissolved in 50 cc. of water and was heated with 3.5 mols of phenylhydrazine acetate. After 1 hour on the water bath an osazone was filtered off which was washed with a few drops of methyl alcohol. 0.25 gm. was recovered. This osazone had an initial  $[\alpha]_D = -56.5^\circ$ , mutarotating to  $-23^\circ$  after 48 hours. The melting point was  $204-205^\circ$ . The osazone was obviously glucosazone.

When oxidized with nitric acid in the usual manner, 1.0 gm. of substance yielded 0.38 gm. of potassium acid saccharate.

0.0495 gm. substance: 0.0174 gm.  $K_2SO_4$ .

Calculated for  $HOOC(CHOH)_4COOK$ . K 15.75 per cent.

Found.

" 15.70 " "

From these experiments one may conclude that part of the mixture is glucose.

### 2. Properties of the Non-Fermenting Sugar.

It was observed that the glucose part of the sugar fraction could be fermented away with yeast; consequently 10.0 gm. of sugar were dissolved in 100 cc. of water and the solution was treated with 20 gm. of Fleischmann's yeast. After 12 hours at  $37^\circ$  the yeast was centrifuged off and to the supernatant liquid was added a small amount of alumina to clear the solution. The mixture was again centrifuged and the clear supernatant fluid was concentrated to small volume *in vacuo*. The soluble yeast dextrans were precipitated with neutral lead acetate. The supernatant liquid was then treated with an excess of basic lead acetate and the precipitate was centrifuged off. The supernatant liquid from this precipitation contained no reducing sugars and was discarded.

The lead salt of the sugar acid thus obtained was suspended in water, and the lead was removed with hydrogen sulfide. After filtering off the lead sulfide, the filtrate yielded 3.5 gm. of an acidic reducing substance. This material had an  $[\alpha]_D = -58.8^\circ$  and a reduction value of 40 per cent calculated as glucose.

The substance appears to be an impure disaccharide acid. From its optical rotation one might suspect the material to be

identical with the aldobionic acid described above, for the optical rotations of both substances are approximately the same. However, this second compound does not give a naphthoresorcinol test, and it therefore cannot be identical with the aldobionic acid previously described.

1.0 gm. of the sugar fraction was dissolved in 50 cc. of water and was treated with 2.0 gm. of yeast. After 12 hours the yeast was removed and the solution was diluted to 100 cc. in a volumetric flask. A similar blank experiment was made, substituting pure glucose for the unknown sugar. After fermentation the first solution showed a reduction of 27 per cent (calculated as glucose on the basis of total weight); the blank solution showed no reduction.

This experiment demonstrates that half of the total sugars in the sugar fraction is fermentable by yeast. The sugar fraction appears, therefore, to be made up from equal parts of glucose (reduction value 100 per cent) and a second disaccharide acid (reduction value 50 per cent).

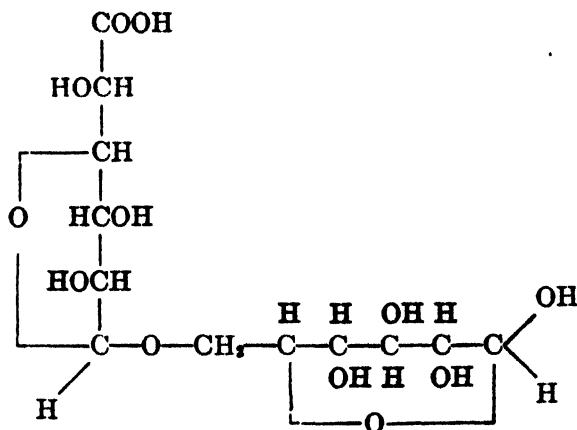
#### DISCUSSION.

The aldobionic acid which forms approximately one-third of the hydrolytic products of the Type A Friedländer specific carbohydrate, corresponds to the formula  $C_{12}H_{20}O_{12}$ . The acid has a reduction value of 50 per cent that of glucose; it contains one carboxyl and one aldehydic group in the molecule. The reducing group is aldehydic as shown by the fact that it may be quantitatively estimated by the method of Willstätter and Schudel. The acid yields glucose on hydrolysis, but when hydrolyzed in the presence of an oxidizing agent saccharic acid is obtained. Since glucose, the hexose half of the aldobionic acid molecule, does not yield saccharic acid under such conditions, one must conclude that this substance is derived from the -uronic acid half of the molecule. This -uronic acid must necessarily be glucuronic acid. The aldobionic acid may be considered therefore as a compound built up from 1 molecule of glucose and 1 molecule of glucuronic acid in such a manner that one aldehyde group and the carboxyl group remain free.

When the aldehydic group of the aldobionic acid is oxidized to a

carboxyl group by means of barium hypiodite, a dibasic sugar acid is obtained which still contains an intact molecule of glucuronic acid. The conclusion which may be drawn is obvious; namely, that the reducing group of the glucuronic acid is protected by chemical combination. The aldobionic acid may therefore be considered as a glucoside of glucuronic acid and glucose. The glucosidic linkage is through the reducing group of the hexuronic acid to one of the carbon atoms of glucose.

The formula



would satisfy the properties of the aldobionic acid which has been described. Whether the linkage is on carbon atom (6) of the glucose molecule or on one of the other carbon atoms is at present unknown.

The hexose and uronic acids as well as their mode of linkage in this new aldobionic acid are the same as in the case of the aldobionic acid from the *Pneumococcus* Type III specific carbohydrate. The two acids differ, however, in their specific optical rotations and in their stability toward mineral acid hydrolysis. These differences may be attributed to differences in the position of the linkage between the glucose and glucuronic acid. The connecting bond in each case probably lies on a different carbon atom of the glucose molecule.

It seems to be extremely significant that two sugar acids which differ only in their spatial configuration, have been isolated from the capsular material of two totally unrelated microorganisms. The significance of these acids will be discussed in a future communication.

The so called sugar fraction which forms the remaining two-thirds of the hydrolytic products of the Type A Friedländer specific carbohydrate, has a reduction value of 75 per cent that of glucose. Half of the sugar which composes this fraction may be fermented away with yeast. The non-fermenting sugar which remains has a reduction value of approximately 50 per cent that of glucose. This sugar appears to be a lactone of a second disaccharide acid. The compound has not been investigated in detail as yet. The fermentable sugar is undoubtedly glucose, since both glucosazone and saccharic acid were isolated in excellent yields.

In conclusion it may be said that the hydrolytic products of the Type A Friedländer soluble specific substance appear to be three sugars; namely, an aldobionic acid, glucose, and a second disaccharide acid. These three sugars are found approximately in the ratio of 1:1:1. If one considers the polysaccharide as a whole, as built up from 2 molecules of aldobionic acid (1 of which is in the form of a lactone) and 1 molecule of glucose, the complex molecule may be represented by the gross formula  $(C_{30}H_{44}O_{26})_x$ . Such a compound should have an acid equivalent of 410 and a carbon and hydrogen content of 43.9 per cent and 5.4 per cent respectively. A comparison of these figures with the observed values in Table I shows a remarkably close approximation.

#### SUMMARY.

1. The soluble specific substance of the Type A Friedländer bacillus yields on hydrolysis an aldobionic acid, glucose, and a second unidentified sugar acid. These compounds occur approximately in the ratio of 1:1:1.

2. A detailed chemical study of the new aldobionic acid has been made, showing it to consist of a molecule of glucuronic acid linked through its reducing group to a molecule of glucose. It is isomeric with an acid derived similarly from the soluble specific substance of Type III pneumococcus.

3. The polysaccharide appears to be a condensate of 2 molecules of aldobionic acid and 1 molecule of glucose.

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## INFLUENCE OF FAT AND CARBOHYDRATE DIETS UPON THE LEVEL OF BLOOD URIC ACID.\*

BY VICTOR JOHN HARDING, KATHLEEN DREW ALLIN, AND  
BLYTHE ALFRED EAGLES.

*(From the Department of Pathological Chemistry, University of Toronto,  
and the Metabolism Ward, Burnside Maternity Wing, Toronto General  
Hospital, Toronto, Canada.)*

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Some time ago Harding, Allin, Eagles, and Van Wyck (1) published in this *Journal* the fact that the level of blood uric acid was invariably raised when the subject under observation was on a high fat diet. They also indicated that the increased level of blood uric acid was in all probability due to decreased excretion. This they inferred from the studies of Cathcart (2), Graham and Poulton (3), and Umeda (4), recorded in the literature, who had noted a decreased excretion of uric acid on high fat diets, and from their own data that carbohydrate feeding immediately succeeding the high fat period was marked by a sharp increase in uric acid excretion for the first 24 or 48 hours. The evidence seemed so conclusive that we almost deemed it unnecessary to pursue the matter further and to present direct proof. Lennox (5), however, without disagreeing with our main contention, that the increased blood uric acid is the consequence of a decreased excretion, notes the omission.

The analytical methods used were the following: non-protein nitrogen, Folin and Wu (6), urea nitrogen, Van Slyke and Cullen (7), uric acid in blood, Benedict (8), uric acid in urine, Benedict (9), CO<sub>2</sub>-combining power, Van Slyke (10), total acetone bodies,

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Van Slyke (11), total urine nitrogen, Kjeldahl-Gunning method (12).

Charts I and II show the excretion of uric acid and the level of the blood uric acid upon ketonuria-producing diets. The subjects G-h-m (three experiments) and F-b-s were those used in the experiments on the determination of the threshold of ketonuria in pregnancy already published in this *Journal* (13), and the uric acid determinations were made at the same time. Subject Y-h-n was also pregnant, but subjects W-t and E-y were normal non-pregnant women. The fat diets were all similar in character being composed of a mixture of cream, milk, and egg white, adjusted to be slightly under the calory requirements of the subject. An example of the composition of such a diet, and as used by subject G-h-m, is to be found in Table I. All eight experiments show that at the commencement of the high fat diet there is a decreased excretion of uric acid. Sometimes the drop in excretion is followed by a sharp rise even while on the high fat diet. In others the low excretion is continued for the duration of the high fat diet. A protein diet subsequent to the high fat diet produced an increased excretion of uric acid with a sharp drop in the level of the blood uric acid, being thus similar to the action of carbohydrate. Our conjecture that the rise of blood uric acid observed under the high fat diet is the result of decreased excretion is thus confirmed. The results of Lennox show that the rise of blood uric acid observed by him in fasting is also accompanied by a decrease in elimination, and the two series of experiments sustain the contention of Folin, Berglund, and Derick (14) that many alterations in the output of endogenous uric acid are brought about by alterations in the level of that substance in the blood.

In Charts I and II are also noted the "total acetone" excretions. In the previous paper it was noted that the rapid increases in blood uric acid occurred in those cases showing a high total acetone excretion, usually over 2 to 3 gm. Of the eight experiments shown in Charts I and II, four in Chart I show increases in blood uric acid; small in amount (4.0 mg. as a maximum) and an excretion of total acetone never exceeding 2.64 gm. per day. The four experiments in Chart II show the level of the blood uric acid raised to 5.5 mg. as a minimum and total acetone excretion

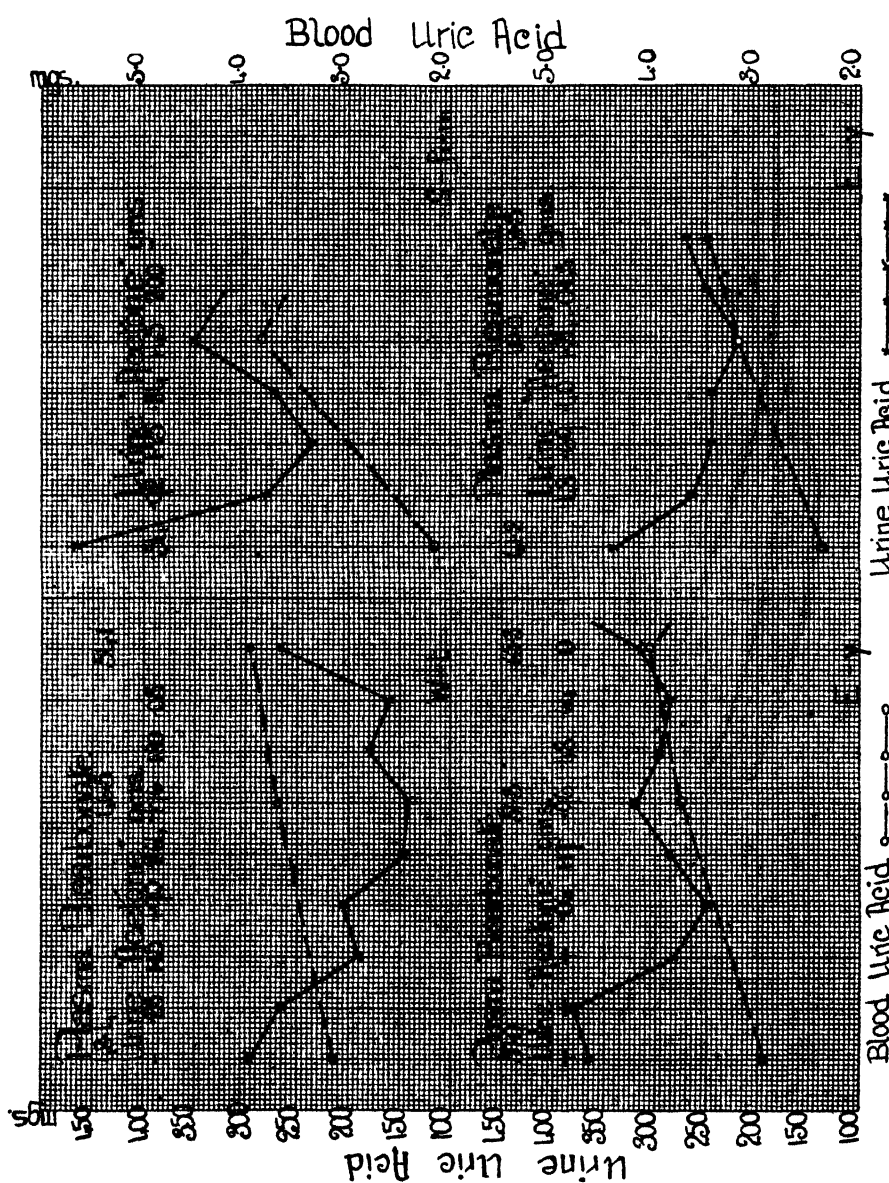


CHART I. Showing the decreased excretion of uric acid and the increased level of blood uric acid on high fat diets. Low "acetone" excretion.

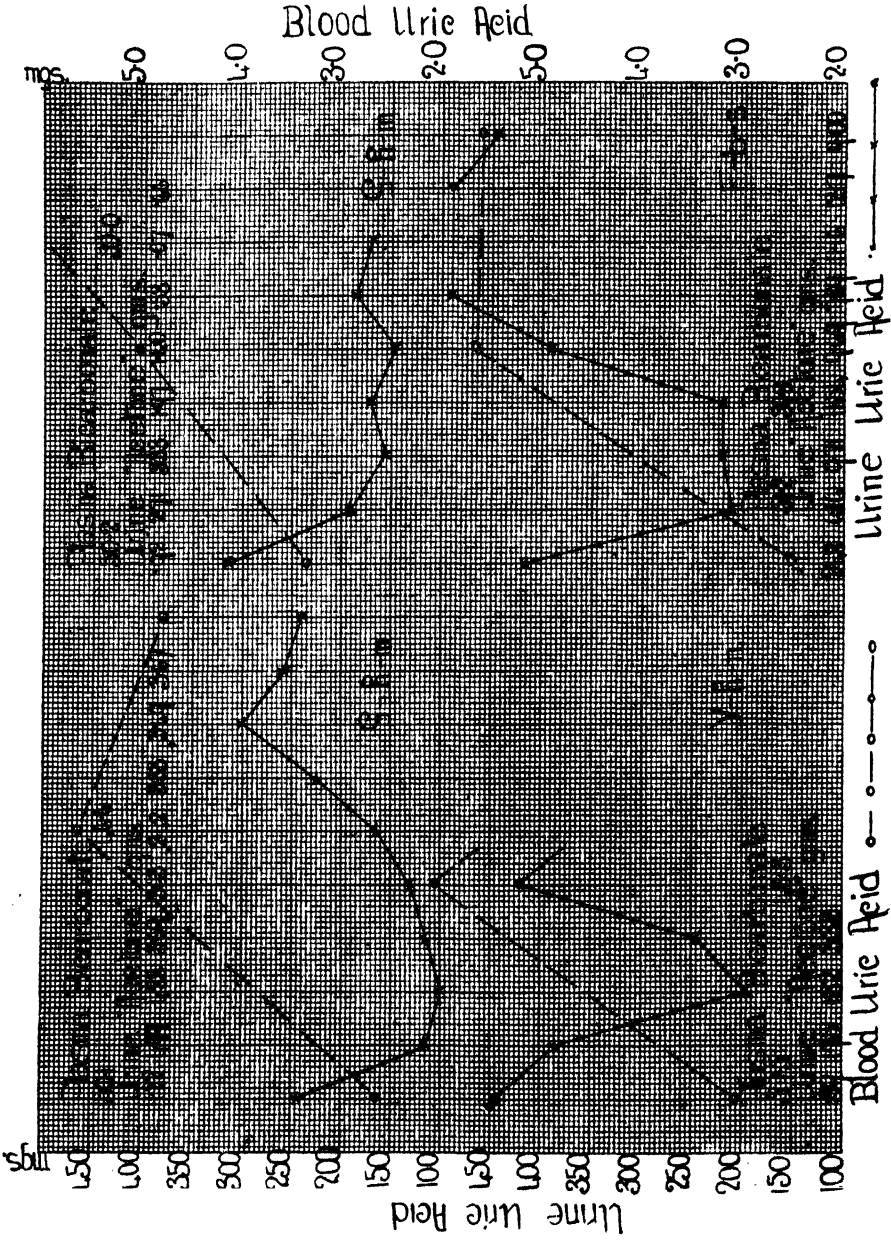


CHART II. Showing the decreased excretion of uric acid and the increased level of blood uric acid on high fat diets. High "acetone" excretion.

TABLE I.  
*Diets Used in Experiments Shown in Charts I to III.*

Diets.....	I	II	III	IV
	gm.	gm.	gm.	gm.
Oatmeal porridge.....		78	90	30 (dry).
Tapioca pudding.....		112	90	
Rice.....		130	95	100
Bread.....		180	155	150
Corn.....				120
Vegetables, 5 per cent.....				80
"    15 "    "    .....				100
Potatoes.....		150	115	330
Beef.....		25		
Chicken.....				40
Banana.....		108	70	50
Orange.....		50	45	60
"    juice.....				100
Grape ".....				400
Baked apple.....				150
Stewed figs.....				150
Raisins.....				40
Jam.....		92	70	40
Sugar.....		48	35	109
Cream, 18 per cent.....			22	
"    32 "    "    .....	320			280
Butter.....		18	21	68
Milk.....	670	130	70	
Egg white.....	85		20	
Tea.....				
Carbohydrate.....	44.5	339.2	312.5	628
Fat.....	129.0	32.1	24.5	150
Protein.....	40.6	39.2	30.8	34
Calories.....	1546	1840	1468	4001

Diet I is the high fat diet used in experiments shown in Charts I and II. Diet II is the carbohydrate diet of subject M-r (Table II). Diet III is the carbohydrate diet of subjects Sh-p and B-k-r (Chart IV). Diet IV is the carbohydrate diet of subject D-k-n (Table III).

of 3 gm. or over on at least one experimental day. Our previous conclusion is thus upheld, though we regard as fortuitous the rather exact agreement of the two sets of figures. We only wish to draw the general conclusion that the height of the blood uric acid in subjects on high fat diets is to be correlated with the extent

of the ketonuria. Lennox has noted during his fasting experiments that: "In those periods in which there were no complicating experimental procedures . . . the curves representing blood uric acid and plasma bicarbonate ran a reciprocal course . . . ." "This reciprocal relationship, however, was only a general one. On various occasions the uric acid of the blood would vary, without coincident variation in the bicarbonate of the plasma." On Charts I and II will also be found the values for the plasma bicarbonate noted in our experiments. With the production of a ketosis there will necessarily be observed a diminution in the plasma bicarbonate: the extent of the lowering of the plasma bicarbonate, however, forms no unerring index to the rise in uric acid. The extent of the ketonuria is a much better guide. Lennox infers from his failure to observe a rise in blood uric acid on administration of  $\text{CaCl}_2$  that ketosis rather than acidosis is the important factor. There the plasma bicarbonate is lowered; the excretion of uric acid however, is increased; the use of  $\text{CaCl}_2$  leads to a loss of body water. Lennox uses this experiment as an irrefutable argument against our explanation that a decreased blood volume might be accountable for the decreased excretion of uric acid which we expected. We thought, however, that we had been careful to point out the inadequacy of this explanation on quantitative grounds, and that it was in search for some more dominating factor that we had noticed the correlation of the extent of the ketonuria and the rise in blood uric acid. We hazarded the suggestion that changes in ionic balance might be responsible, but such changes must evidently be of a very definite character when opposite results are obtained by the lowering of the plasma bicarbonate through the agency of  $\text{CaCl}_2$  or "acetone."

The discrepancies, however, are those of explanation rather than of experiment. The effect of the ketosis in either fasting or high fat feeding, results in a decreased excretion of uric acid in the urine with an increase in the blood uric acid. The effect of a high carbohydrate diet is, however, not such a matter of unanimity. Folin, Berglund, and Derick noted that the level of blood uric acid on low protein purine-free diets was higher than on high protein purine-free diets in the same individuals. The low protein diet was of mixed character, which, according to their tables,

led to an excretion of 6.5 gm. of N as a maximum after equilibrium had been reached and giving an average plasma uric acid of 4.9 mg. per 100 cc. The same subjects on high protein diet showed urinary N figures of 13.3 to 19.6 gm. and showed an average plasma uric acid of 3.4 mg. per 100 cc. The distribution of the remaining calories between carbohydrate and fat is not mentioned, but it is presumed that the latter did not preponderate. A large amount of fat in the low protein diet would have made an unusual dietary to which special attention would undoubtedly have been drawn. We mention this possibility because although the rise in blood uric acid on fat diets has, as yet, only been observed in ketosis-producing diets, an experiment of Umeda might well be interpreted that a rise in blood uric acid could be observed on low protein-high fat diets, in which the fat was not at a high enough level to produce ketosis. The diminished uric acid excretion though small, observed in Umeda's (4) Experiment I (Table IB, p. 423), if continued for a sufficient length of time, might produce a rise in blood uric acid. Harding, Allin, and Van Wyck (15) had been unable to find any significant variation in blood uric acid on low protein diets contrasted with high protein diets, in which the N intake was respectively 4.8 and 18.8 gm. and in which the greater balance of the remaining calories arose from carbohydrate. In the course of some experiments of Harding and Montgomery (16) on the puerperium in which diets, high in carbohydrate, had been used, the excretion of uric acid in the urine and its level in the blood were also observed, with the hope of throwing light upon the discrepancy. Of the six experiments we have selected two to report in detail, as in both these cases the baby died at, or shortly after, birth, so the puerperium was not complicated by lactation. We may state, however, that the results on the other four normal cases are exactly similar in nature, and lactation possesses no observable influence over the course of the purine metabolism under these conditions.<sup>1</sup> The purines of the human uterus and their metabolism during involution do not appear to have been studied from a chemical

<sup>1</sup> The blood uric acid values in mg. per 100 cc. for the lactating subjects on similar diets showed the following variations: G-h-m 3.8, 3.9, 3.5, 4.0 (14 days), B-l-n 4.1, 3.8, 3.1, 4.8, 3.4, 3.1 (11 days), W-r 5.0, 4.1, 4.8 (8 days), M-l-r 3.6, 4.6, 4.6, 4.4, 4.2, 4.7 (13 days).



standpoint. In no case, however, were we able to observe evidence of a nuclear breakdown in an increased output of endogenous uric acid during the period of acute involution.

The results on the two selected cases are shown in Tables II and III. The experiments were continued for a period of 13 and 18 days respectively, a length of time amply sufficient to make evident any definite change. The diets were purine-free and supplied 23 and 53 calories per kilo (Table I). The second sub-

TABLE II.

*Excretion of Uric Acid in the Puerperium on Carbohydrate Diet and Level of Blood Uric Acid.*

Case M-r. Normal puerperium. Low protein diet, 1840 calories; N intake 5.6 gm. per day.

Date.	Urine (per day).		Blood (100 cc.).		
	Total N.	Uric acid.	Non-protein N.	Urea N.	Uric acid.
	gm.	mg.	mg.	mg.	mg.
Oct. 12			20.0	5.0	3.0
" 15	8.03	642.0			
" 16	11.12	655.6			
" 17	9.84	625.2			
" 18	9.01	757.8			
" 19	7.31	614.8			
" 20	8.57	703.3	25.0	5.85	3.7
" 21	8.81	694.1			
" 22	8.00	716.4			
" 23	7.67	600.0			
" 24	7.10	609.4			
" 25	6.04	569.9			
" 26	6.95	555.1	22.5	8.4	3.4
" 27	4.86	380.5			

ject was thus supplied with a large excess of calories and was subject D-k-n of the experiments of Harding and Montgomery. The N intake was low in both cases, being 5.6 and 7.0 gm. respectively. The urinary N averaged 7.8 gm. in the first and 4.3 in the second subject. The first was losing N while the second was in positive N balance. The fat content of the diet in both was low, the preponderating amount of calories being drawn from carbohydrate. The effect of the dietary upon the N metabolism

is also reflected in the low urea N in the blood of both cases. The blood uric acid shows no appreciable alteration as a result of the diet in either case. Apparently the conditions were such as would lead to a repetition of the results of Folin, Berglund, and Derick, yet the discrepancy remains and we have merely confirmed our previous findings. We should feel therefore that

TABLE III.

*Excretion of Uric Acid in the Puerperium on Carbohydrate Diet and Level of Blood Uric Acid.*

Case D-k-n. Normal puerperium. Low protein diet, 4000 calories; N intake 7.0 gm. per day.

Date.	Urine (per day).		Blood (100 cc.).		
	Total N.	Uric acid.	Non-protein N.	Urea N.	Uric acid.
	gm.	mg.	mg.	mg.	mg.
Apr. 24	6.96	556.0			
" 25	6.96	342.1			
" 26	4.02	417.7			
" 27	4.27	446.8	12.86	5.86	3.2
" 28	5.02	473.9			
" 29	4.46	517.2	14.8	5.98	3.3
" 30	3.67	417.7			
May 1	4.19	456.0	18.6	6.76	3.0
" 2	3.74	337.1			
" 3	3.96	443.8			
" 4	3.57	392.3	17.9	7.78	3.1
" 5	4.45	477.4			
" 6	4.17	489.3	10.6	4.66	4.1
" 7	4.17	520.1	16.8	4.66	3.1
" 8	3.82	454.5	16.2	6.99	3.1
" 9	3.25	460.3			
" 10	3.51	460.2			
" 11	3.65	471.9			

we are in a position to uphold the conclusion that low protein-high carbohydrate diets have no effect in raising blood uric acid, and that such diets might be used with equal value to high protein diets, by gouty subjects. We should, however, be much more confident in our assertion had we not been able to observe the levels of the blood uric acid in two normal pregnant subjects for a period of 5 months under the influence of various diets. The

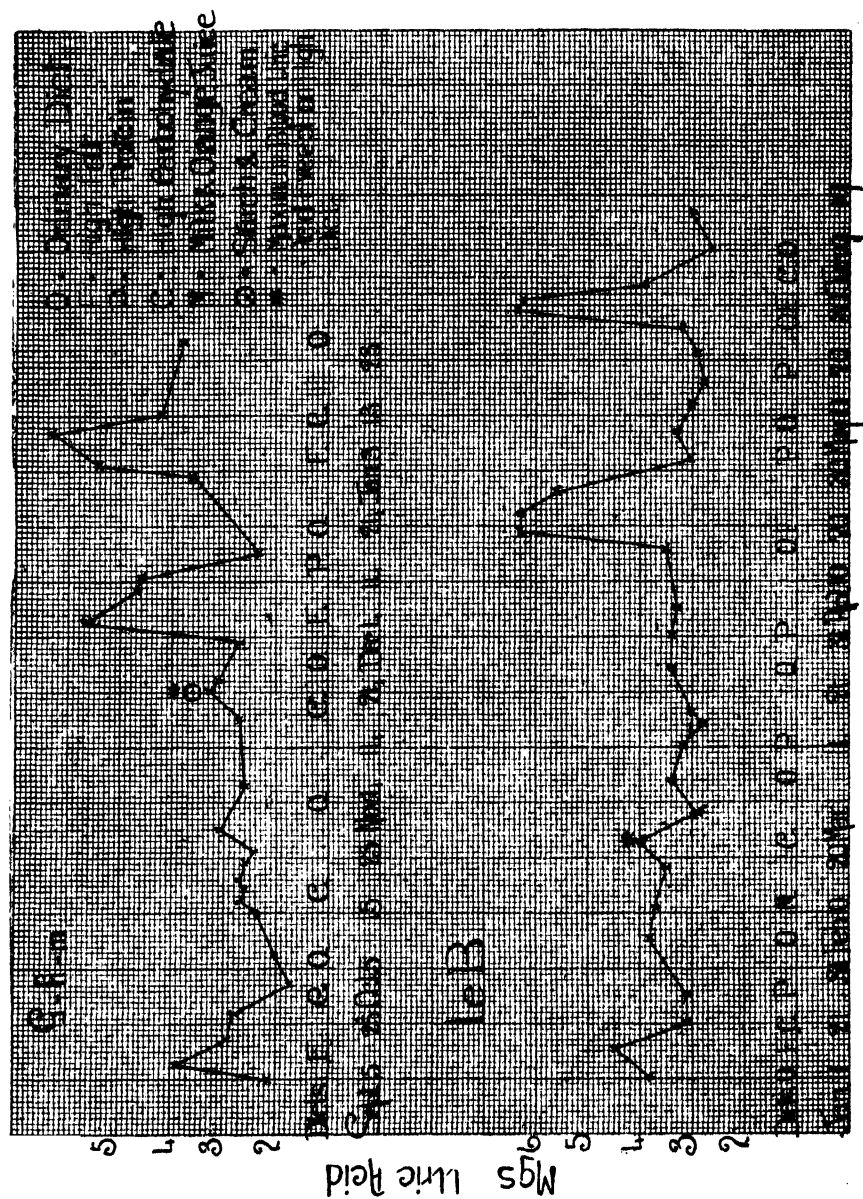


CHART III. Showing effect of fat, protein, and carbohydrate diets on level uric acid.

results are shown in Chart III. In both cases it is to be noticed that the highest blood uric acids (marked with asterisk) observed over a period of nearly 6 months occurred on carbohydrate diets

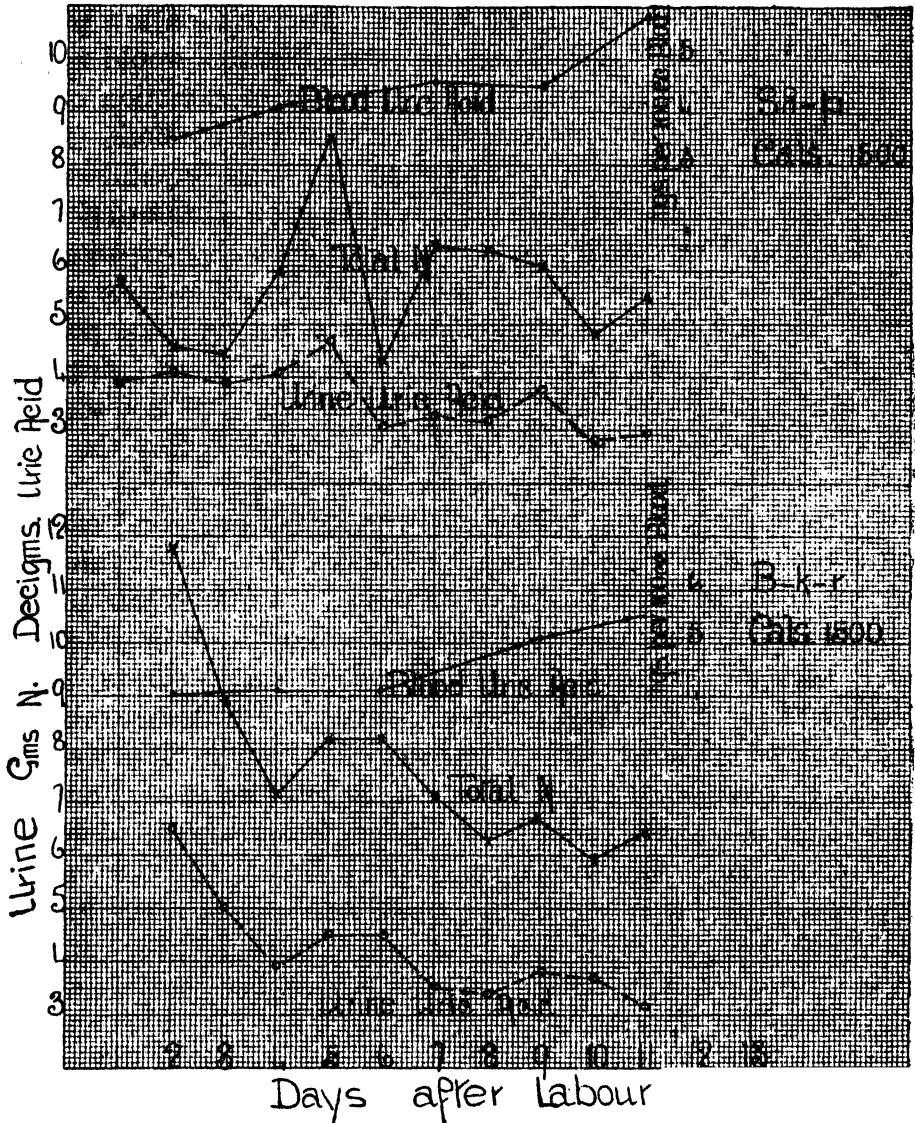


CHART IV. Showing uric acid excretion in puerperium on high carbohydrate diets and influence on level of blood uric acid (toxemias).

(exclusive of the results on high fat diets). In case G-h-m the carbohydrate diet was the Folin starch and cream diet with the addition of a little sugar and 3 gm. of NaCl. In case Le B the

diet was mixed. Neither diet was continued for a long period of time. Such results, taken in conjunction with those of Folin, Berglund, and Derick, can scarcely be accidental, and can only be interpreted by the assumption that the determining factor in the occasionally observed rise in blood uric acid on low protein diets is other than carbohydrate. The situation would appear to be similar to that observed on the level of excretion of uric acid on high and low protein diets as observed by Folin (17) in contrast with Siven (18). The term acid-base equilibrium at once occurs to the mind again as an explanation, but until changes in the blood uric acid are more controllable by experimental alteration of the ionic balance than at present, it is wiser not to formulate any hypothesis. Lennox has discussed some possible factors in uric acid excretion and retention without being able to effect their correlation. Okey and Erikson (19) in discussing their results on women are inclined to the view that the ionic balance is the predominating factor.

In contrast with the behavior of our formal puerperal subjects on carbohydrate diets we show in Chart IV the blood uric acid of two toxemia cases, both non-lactating. The diet was similar in character to that used in case M-r, but contained only 4.2 gm. of N with the majority of the calories from carbohydrate; in no sense was the diet a high fat diet (Table I). Both cases show a rising blood uric acid. The difference here may be clearly stated as due to the altered kidney function in these two cases. Subject B-k-r had an acute toxemia of the convulsive type, while the history of Sh-p suggested a previous nephritis. In view of the findings on the increase in uric acid in blood in interstitial or glomerulonephritis of Myers, Fine, and Lough (20), the contrast shown by the two toxemias and the normal subjects in our experiments is of interest.

#### SUMMARY.

The increase in blood uric acid observed on high fat diets is correlated with decreased excretion.

Low protein-high carbohydrate diets of mixed origin fail to raise the level of blood uric acid in the normal puerperium.

Similar diets showed raised blood uric acid in the puerperium following toxemia.

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# EFFECTS OF DIALYSIS AND OF ETHER EXTRACTION ON THE DIFFUSIBILITY OF CALCIUM IN HUMAN BLOOD SERUM.

By ROBERT F. LOEB AND EMILY G. NICHOLS.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York.)

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## INTRODUCTION.

Rona and Takahashi (1) first demonstrated the unequal distribution of calcium between blood serum and an aqueous solution separated by a collodion membrane. These investigators, and many others since, have attributed the unequal calcium concentration at equilibrium to the formation of undissociated and non-dialyzable Ca proteinate because the Ca content of the serum was greater than that of the aqueous solution. Cameron and Moorhouse (2) believe that substances non-protein in character cause these discrepancies, and that the same non-diffusible substances account for the fact that the concentration of Ca is greater in blood serum than in spinal fluid.

In experiments reported last year, it was shown (3, 4) that the Donnan equilibrium accounts in part for the fact that the Ca concentration is greater in a serum than in its dialysate, but there remains a discrepancy between the observed Ca concentrations of the dialyzed sera at equilibrium and the theoretical concentration (as determined by the Donnan law from the concentrations of Ca in the dialysates). Marrack and Thacker (3) have assumed this discrepancy to be due to the formation of unionized Ca-protein compounds as a result of dialysis experiments conducted on protein solutions prepared from horse serum by ether and alcohol precipitations in the cold or by a preliminary dialysis of the horse serum until it was Ca-free. Hastings, Murray, and Sendroy (5) conclude from studying the equilibria between sera and solid calcium carbonate and phosphate that there must be Ca-protein compounds formed in serum and that ionic activity "may play some rôle." The present writers (4, 6) have suggested that there may be complex Ca-protein ions formed, analogous to complexes assumed by Northrop and Kunitz (7) to exist in the case of zinc and proteins.

The present work was undertaken in an endeavor to add evidence in support of the idea that the discrepancy between observed



TABLE I.  
*Dialysis Equilibria of Calcium in Modified Human Blood Sera.*

Serum No.	Modification of serum.	Calculated [Ca] of serum in sacs. mM	Deviation from observed [Ca] of serum. per cent	Observed [Ca] serum mM	Observed [Ca] aqueous solution. mM	[Ca] serum [Ca] aqueous solution	Observed [Cl] serum mM	Observed [Cl] aqueous solution. mM	[Cl] serum [Cl] aqueous solution	pH of aqueous solution.	Protein concentration of serum in sacs. per cent	[Ca] of original serum mM
1	Whole serum.....	1.37	66	2.27	1.13	2.01	121.2	133.8	1.10	7.80	6.14	2.04
	".....	1.46	51	2.21	1.25	1.77	122.5	132.8	1.08	7.30	5.86	
	".....	1.42	60	2.27	1.22	1.86	122.6	132.5	1.08	7.90	5.72	
	Dialyzed.....	1.27	43	1.82	1.09	1.67	123.2	132.9	1.08	7.35	5.65	
	".....	1.44	17(?)	1.69	1.19	1.42	122.8	135.2	1.10	7.30	5.69	
2	Ether-extracted.....	1.59	53	2.44	1.29	1.89	120.9	133.6	1.11	7.20	6.07	1.93
	Whole serum.....	1.58	27	2.00	1.35	1.48	117.9	127.9	1.08	7.50	5.70	
	Dialyzed.....	1.47	20	1.77	1.31	1.35	121.2	128.3	1.06	7.35	4.53	
	".....	1.46	19	1.73	1.27	1.36	120.3	128.2	1.07	7.20	4.87	
	Ether-extracted.....	1.51	42	2.15	1.29	1.67	118.6	127.3	1.08	6.80	5.56	
3	Whole serum.....	1.45	50	2.18	1.24	1.76	117.4	127.1	1.08	7.60	5.64	2.31
	Dialyzed.....	1.37	35	1.85	1.17	1.58	119.2	128.4	1.08	7.30	5.51	
	".....	1.52	45	2.20	1.28	1.72	117.4	127.5	1.09	7.65	5.42	
	Ether-extracted.....											
4	Whole serum.....	1.42	33	1.89	1.24	1.53	122.6	131.6	1.07	7.30	5.61	2.15
	Dialyzed.....	1.38	22	1.69	1.18	1.43	122.7	132.9	1.08	7.20	5.27	
	".....	1.33	66	2.20	1.12	1.98	122.6	133.2	1.09	7.40	5.48	
	Ether-extracted.....											
5	Whole serum.....	1.56	55	2.37	1.34	1.77	122.5	132.4	1.08	7.60	6.71	2.37
	Dialyzed.....	1.53	31	2.00	1.31	1.53	124.4	134.2	1.08	7.40	6.16	
	Ether-extracted.....	1.54	70	2.61	1.32	1.98	123.3	133.8	1.08	7.50	6.74	

6	Whole serum.....	1.54	32	2.03	1.30	1.56	119.2	130.0	1.09	7.65	6.10	2.41
	Dialyzed.....	1.46	29	1.89	1.23	1.54	120.7	131.5	1.09	7.75	6.02	
6A	Whole serum.....	1.35	58	2.14	1.16	1.84	121.8	131.1	1.08	7.50	5.58	2.41
	Dialyzed.....	1.32	37	1.81	1.12	1.62	122.7	132.6	1.08	7.30	5.67	
7	Whole serum.....	1.53	21	1.85	1.36	1.36	123.2	131.2	1.06	7.25	5.12	2.40
	Dialyzed.....	1.34	22	1.74	1.22	1.42	123.2	129.2	1.05	7.25	5.30	
8	Whole serum.....	1.51	38	2.08	1.29	1.61	122.7	131.2	1.08	7.65	5.04	2.50
	Dialyzed.....	1.48	20	1.78	1.27	1.40	123.6	132.3	1.08	7.30	5.04	
8A	Whole serum.....	1.32	52	2.01	1.20	1.67	125.0	130.8	1.05	7.20	4.95	2.50
	Dialyzed.....	1.36	26	1.72	1.19	1.45	124.5	133.2	1.07	7.35	5.12	
9	Whole serum.....	1.33	46	1.94	1.14	1.70	124.9	134.3	1.08	7.45	5.94	2.38
	Dialyzed.....	1.42	51	2.14	1.20	1.78	122.0	133.5	1.09	7.65	5.85	
10	Whole serum.....	1.49	38	2.05	1.25	1.64	123.4	134.2	1.09	7.00	6.08	2.58
	Dialyzed.....	1.50	27	1.91	1.22	1.57	123.1	135.6	1.10	7.35	6.04	
	Ether-extracted.....	1.47	48	2.17	1.24	1.75	123.3	134.2	1.09	8.00+	5.84	
11	Whole serum.....	1.52	43	2.18	1.28	1.70	123.8	134.8	1.09	7.80	6.38	2.66
	Dialyzed.....	1.37	35	1.85	1.17	1.58	125.3	135.6	1.08	7.40	6.34	

$$\frac{\text{* Observed [Ca] serum - calculated [Ca] serum}}{\text{Calculated [Ca] serum}} \times 100.$$

Average deviation from calculated [Ca] serum with whole serum = +45 per cent.
" " " " "
" " " " dialyzed serum = +30 per cent.
" " " " ether-extracted serum = +54 per cent.

and calculated Ca concentrations in sera is dependent upon the protein fraction of the serum and not upon the lipoids or some other substance (of the type of citrate) combined with calcium.

#### EXPERIMENTAL.

Sera were obtained from patients most of whom were suffering from hypertension or cardiac insufficiency. Each serum was then divided into three lots. The first lot or whole serum was set aside until the preliminary treatment of the other two fractions was completed. Lot 2 was rendered Ca-free by prolonged dialysis against large amounts of 0.8 per cent NaCl at pH 7.4. Fraction 3 was extracted twice by prolonged shaking with equal volumes of ether. It was found that this process removed 70 to 80 per cent of the total lipoids present. The whole sera and those extracted with ether were diluted with 0.8 per cent NaCl solution at pH 7.4 in order to make the protein concentrations comparable with those of the samples of sera dialyzed Ca-free. The three lots of sera were then placed in collodion sacs which were immersed in bottles containing 250 cc. of 0.8 per cent NaCl at pH 7.4 to which was added sufficient  $\text{CaCl}_2$  to make the Ca concentration of the outside fluid 1.45 millimols per liter. Dialysis continued for 24 to 48 hours and was carried on in an ice box to inhibit bacterial growth. Details of the dialysis procedure and of the analytical methods employed have been reported elsewhere (4).

#### DISCUSSION.

From Table I (fourth column) it may be seen that at equilibrium the concentrations of Ca in all three lots of each serum are invariably greater than the theoretical Donnan values as calculated from the equation:

$$r = \frac{[\text{Cl}] \text{ dialysate}}{[\text{Cl}] \text{ serum}} = \frac{\sqrt{[\text{Ca}] \text{ serum}}}{\sqrt{[\text{Ca}] \text{ dialysate}}}$$

where  $r$  = the Donnan ratio.

In these calculations, it has been assumed that the analytical concentrations of Cl in serum and dialysate may be used as fairly accurate measures of the Donnan ratio. Possible deviations due to differences in ionic activities or to the presence of "organic chlorides" (8) have been neglected.

It becomes clear that the lipoids of serum cannot be responsible for the discrepancies between theoretical and observed [Ca] values in the contents of the collodion sacs, for the discrepancies appear to be on an average of about 10 per cent greater than in the case of untreated sera. The significance of this 10 per cent difference is doubtful.

While the average values for [Ca] in the sera first dialyzed Ca-free are in general low, and therefore approach somewhat more closely the theoretical Donnan figure, in some cases the discrepancies are equal to or even greater than those in the corresponding untreated sera. It is possible that the concentrations of Ca in the sera initially dialyzed Ca-free tend to be low as a result of partial denaturization of proteins through prolonged dialysis.

#### CONCLUSIONS.

1. A comparative and simultaneous study of Ca concentrations at equilibrium in a dialysis system has been made on human sera divided into the following fractions: (a) whole serum, (b) the same serum rendered Ca-free by preliminary dialysis, (c) the same whole serum extracted with ether.

2. The observed Ca concentrations in all three fractions are considerably higher than can be accounted for by the Donnan equilibrium. The deviation appears to be slightly greater than in the whole sera in the case of the ether-extracted sera and slightly lower in the fractions rendered Ca-free by preliminary dialysis.

3. The results substantiate the idea that there are unionized Ca-protein complexes in human sera and that if there are any non-protein factors present their influence must be quantitatively very small.

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## THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL AND PHYTOSTEROL.

### VIII. THE ACTIVATION OF STEROL FRACTIONS BY ULTRAVIOLET IRRADIATION.

BY ALFRED F. HESS AND R. J. ANDERSON.

*(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, and the Biochemical Laboratory, New York Agricultural Experiment Station, Geneva.)*

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It is now established that cholesterol develops antirachitic properties following irradiation with ultra-violet light. Of late, however, it has come to be questioned by various workers in this field whether it is the cholesterol itself or some associated or contaminating substance which undergoes activation in the course of this process. This doubt was expressed recently in communications by Rosenheim and Webster (1), by Heilbron and his co-workers (2), and by Hess and Windaus (3), who found that various sterols which they had expected to become active failed to develop antirachitic properties. The fact that less than 1 per cent of cholesterol becomes active as the result of irradiation would also suggest this interpretation. Further research showed that failure to activate was associated intimately with the bromination to which the sterols had been subjected in the course of purification. Not only did cholesterol, purified by bromination, fail to develop antirachitic properties, but it failed to show the characteristic absorption spectrum of ordinary cholesterol or a decrease in absorption following irradiation—a phenomenon described some time ago by Hess and Weinstock (4) as characteristic of irradiated cholesterol, and corroborated and amplified by Schlutz and Morse (5), by Heilbron *et al.* (6), and by Pohl (7).

The pursuit of this line of investigation led to the discovery that it is probably an allied sterol, ergosterol, which is removed or destroyed in the course of bromination, and that this substance is the

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precursor of the antirachitic factor. This conclusion was reached by Windaus and Hess (8) and by Rosenheim and Webster (9), who more recently have reported that overirradiation or treatment with charcoal also serves to destroy the "provitamin." Ergosterol is an optically active sterol, unsaturated—having three double bonds—and containing a hydroxyl radical. Its molecule therefore possesses the two factors which have been found to be closely linked with the activation of cholesterol derivatives. When ergosterol prepared from yeast was irradiated by the mercury vapor lamp, then dissolved in linseed oil and fed to rats, it was found that healing of the rachitic bone was brought about when as little as 0.001 mg. per capita was given daily, whereas when irradiated cholesterol was fed approximately 1 mg. had been required to initiate healing. Rosenheim recently has reported that even 0.0001 mg. exerts definite antirachitic action, and that the specific alteration in ergosterol can be observed by means of the spectral absorption method in still greater dilutions. Although it may be considered as proved that ergosterol develops antirachitic potency to a remarkable degree following irradiation, it has not been demonstrated that it is the only sterol which possesses this remarkable characteristic. In this connection it should be borne in mind that it was shown recently by Anderson (10) that cholesterol preparations obtained from various sources manifest differences in their physical properties, which probably are to be ascribed to an admixture of isomeric sterols. Following this trend of thought we prepared some pure sterol preparations to ascertain whether any of the fractions would show antirachitic activity following irradiation. Fractions of this kind, prepared from corn oil, have been described recently by Anderson and Shriner (11).

During the progress of these chemical experiments it was observed that the properties of the various sterol fractions showed decided differences not only in optical rotation but also in stability and in solubility. The top fractions, which have been designated  $\beta$ - and  $\gamma$ -sitosterol were much more stable and less soluble than the bottom fraction which was called  $\alpha$ -sitosterol. The  $\beta$  and  $\gamma$  fractions could be kept for months in ordinary glass-stoppered specimen bottles without showing any change in color or melting point. On the other hand, the  $\alpha$ -sitosterol fractions were more sensitive when kept under similar conditions, acquiring after a

few weeks a yellow color and a peculiar rancid odor. It was quite evident that this fraction was easily changed when in contact with atmospheric oxygen. In view of this comparative instability, it was believed that after irradiation the  $\alpha$ -sitosterol would exhibit greater physiological activity than would the  $\beta$ - and  $\gamma$ -sitosterol.

The chemical work on the separation of the sterols mentioned above was completed early in 1926. Due to various circumstances, however, it was not possible to submit these compounds to physiological experiment until early in 1927. At the time the  $\beta$ - and  $\gamma$ -sitosterols were pure white and odorless, whereas the  $\alpha$ -sitosterol had a yellowish color and a pronounced rancid odor. These fractions were irradiated, dissolved in linseed oil, and fed by pipette to rats, by the technique which has been followed in this laboratory in the course of previous investigations, the only difference being that curative, as well as preventive tests were employed. In these experiments 2.5 mg. of each fraction were fed daily. This amount is the standard quantity which has been given in the course of our cholesterol experiments. It is, however, an exceedingly large dose when we bear in mind the minute quantities of irradiated ergosterol which suffice to bring about an antirachitic action.

The results of the irradiation and feeding experiments in January, 1927, indicated that all three of the sterols named above were incapable of acquiring antirachitic properties by exposure to ultraviolet light. Since the  $\beta$ - and  $\gamma$ -sitosterols had been purified by means of the dibromo compounds we did not expect, in view of the work of Windaus and Hess and of Rosenheim, that these fractions would show any protective action against rickets.

The fact that  $\alpha$ -sitosterol which had been purified without bromination could not be activated by irradiation after the preparation had stood for about 1 year would indicate that profound changes in the molecule had been induced by spontaneous oxidation.

Fresh preparations of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sitosterols were made from corn oil by the following method, and once more tested.

#### *Preparation of Unsaponifiable Matter and Sitosterols.<sup>1</sup>*

1 kilo of Mazola corn oil was saponified by heating it with alcoholic potassium hydroxide. The soap solution was largely diluted

<sup>1</sup> We are indebted to Dr. R. L. Shriner, New York Agricultural Experiment Station, Geneva, for kindly preparing these sterol fractions.



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with distilled water and extracted with four portions of ether. The ethereal solution was washed several times with water, filtered, and the ether was removed by distillation. The residue was again boiled with alcoholic potassium hydroxide for 1 hour, diluted with water, and extracted with ether. After the ethereal solution was washed with water, it was filtered and the ether was distilled.

The residue which weighed 12 gm. was dissolved in a small quantity of acetone and the solution was cooled for some time in a freezing mixture. The crystalline sterols that separated were filtered off, washed with a little ice-cold acetone, and allowed to dry in the air at room temperature. This treatment with acetone served to eliminate most of the non-crystalline portion of the unsaponifiable matter. The nearly white crystals that were obtained weighed 9.0 gm.

### *Preparation of $\beta$ - and $\gamma$ -Sitosterol.*

The crude crystalline sterols were recrystallized five times from 95 per cent alcohol. The final crystals, which were snow-white, were filtered off, washed with cold alcohol, and dried in the air.

The mother liquors were saved and used for the preparation of  $\alpha$ -sitosterol.

The top fraction of the sterols which weighed 4 gm. was acetylated by boiling with acetic anhydride for 2 hours. The reaction mixture was diluted with water and the acetyl derivative which precipitated was filtered off, washed with water, and dried in a vacuum desiccator over sulfuric acid. After the substance had been twice recrystallized from alcohol it was dissolved in ether and the solution was mixed with an excess of 5 per cent bromine in glacial acetic acid. The solution, after it had stood for 2 hours at room temperature, was poured into water and the precipitated dibromo compound was filtered off, washed with water, and dried in a vacuum desiccator. The dibromide was dissolved in 200 cc. of 95 per cent alcohol and the solution was mixed with 10 cc. of glacial acetic acid and 10 gm. of zinc dust and the mixture was refluxed for 2 hours. More acetic acid and zinc dust were then added and the boiling was continued for another hour. The hot solution was filtered to remove the undissolved zinc and the acetyl derivative was precipitated by adding water. The precipitate was filtered off, washed with water, and dried. The product was

recrystallized five times from alcohol and then saponified by boiling with alcoholic potassium hydroxide. The reaction mixture was precipitated by adding water. The free sterol that separated was filtered off, washed with water, dried, and recrystallized three times from alcohol when snow-white crystals were obtained. The substance melted at  $140\text{--}141^\circ$  (corrected). On drying in a vacuum at  $78^\circ$  over phosphorus pentoxide the loss in weight was 3.08 per cent. The rotation in chloroform solution using sodium light was  $[\alpha]_D^{25} = 35.51^\circ$ .

This product represents a mixture of  $\beta$ - and  $\gamma$ -sitosterol and also contains a small amount of dihydrositosterol and a trace of stigmasterol because previous investigations have shown that the two latter sterols are present in corn oil.

#### *Preparation of $\alpha$ -Sitosterol.*

The mother liquors obtained on recrystallizing the top fraction of the crude sterol, as mentioned previously, were concentrated to small volume. The yellowish crystals that separated on cooling were converted into the acetyl derivative by boiling with acetic anhydride. The acetyl derivative was treated with norit and recrystallized several times from small volumes of alcohol until it was colorless. The substance was then saponified by boiling with alcoholic potassium hydroxide and the free sterol was isolated and recrystallized from alcohol. The product formed colorless crystals which, after drying in the air, weighed 0.5 gm. The substance melted at  $134\text{--}136^\circ$  (corrected) and in chloroform solution the rotation was  $[\alpha]_D^{20} = 23.86^\circ$ .

As may be noted in Table I the  $\alpha$ -sitosterol was found to be strongly antirachitic, whereas the  $\beta$ - and  $\gamma$ -sitosterols were inactive. It is evident therefore that the substance which is capable of activation by ultra-violet light is present only in the  $\alpha$ -sitosterol; in other words, in the bottom fraction which has not been brominated. If any of this substance had been present in the less soluble portions, constituting the  $\beta$ - and  $\gamma$ -sitosterol, it was destroyed during bromination.

The question must be considered still open as to the true nature of the factor or factors which are activated by irradiation. As stated, it has been shown by Windaus and Hess and by Rosenheim

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that ergosterol can function as the "provitamin" or precursor of the antirachitic factor and that irradiated ergosterol possesses a far greater antirachitic activity than any other sterol thus far tested. Whether traces of ergosterol are present in corn oil is not known, and it cannot therefore be definitely stated whether in our experiments ergosterol was the substance extracted and activated. If

TABLE I.  
*Antirachitic Activity of Fractions of Sitosterol Prepared from Corn.*

Rat No.	Weights.	Rickets-producing diet.	Rickets previous to test.	Supplement to diet.	Subsequent healing (9 days).	Blood P (inorganic).
	gm.					mg.
11389	28-30	Low phosphorus No. 84.	Slight.	2.5 mg. $\alpha$ -sitosterol irradiated $\frac{1}{2}$ hr. at 1 ft.	Slight or moderate.	2.6
11382	30-32		Moderate.		Marked.	
11381	30-30		"		Moderate.	
11390	30-30		"		"	
11580	50-60		"		Marked.	
11581	40-44		"		Slight.	
11587	40-42		"		Marked.	
11588	46-54		"		Almost complete.	
11383	32-26	Low phosphorus No. 84.	Moderate.	2.5 mg. ( $\beta$ - and $\gamma$ -) sitosterol irradiated $\frac{1}{2}$ hr. at 1 ft.	Slight or moderate.	1.8
11385	28-24		"		No.	
11387	30-30		"		"	
11388	30-32		"		"	
11594	48-50		"		"	
11595	42-50		"		"	
11597	44-50		"		"	

ergosterol is the sole antirachitic precursor, it is evident that this sterol must be universally present in all fats of animal and of plant origin that are capable of activation by ultra-violet radiations. This is an exceedingly broad conception. Considerable further chemical and biologic investigations will be necessary before it can be decided whether certain sterols other than ergosterol can contribute to the antirachitic activity of irradiated material.

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# **A GASOMETRIC MICRO METHOD FOR DETERMINATION OF IODATES AND SULFATES, AND ITS APPLICATION TO THE ESTIMATION OF TOTAL BASE IN BLOOD SERUM.**

**By DONALD D. VAN SLYKE, ALMA HILLER, AND  
KNUD C. BERTHELTSEN.**

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

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Fiske (1) developed for determination of the total base in urine a procedure in which the phosphates were removed with iron and the residue was ignited with sulfuric acid, turning all the bases into sulfates. The amount of base, in combining equivalents, was then ascertained by determining, by the benzidine titration, the  $\text{SO}_4$  content of the sulfate mixture. The method is accurate, adaptable to micro modifications, and avoids the errors to which the micro methods for sodium and potassium are as yet subject, because of inconstancy in the antimonate and cobalt-nitrite precipitates.

Van Slyke, Wu, and McLean (2) applied the principle of Fiske's method to the estimation of total base in blood after the proteins had been removed by trichloroacetic acid, a method of removal which, as will be shown, introduces a small but appreciable error. They weighed the  $\text{SO}_4$  as  $\text{BaSO}_4$ , using the filtrate from 5 cc. of blood. Stadie and Ross (3) studied the conditions necessary to modify the procedure for micro analyses by the benzidine method, and obtained good results with 1 cc. portions of serum.

In the present paper a gasometric micro method for sulfate determination is presented, by which the alkali sulfates from 0.16 cc. of serum can be determined as exactly, and somewhat more conveniently, than the sulfates from 1.00 cc. of serum by the benzidine procedure. The gasometric method is based on two reactions. The alkali sulfates are first shaken with an excess of

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pulverized barium iodate. This is classed as an insoluble salt, but barium sulfate is so much more insoluble that a double decomposition occurs, iodate going into solution and sulfate being precipitated.



The mixture is then filtered through a dry filter, and an aliquot of the filtrate is pipetted into the manometric gas apparatus (4, 5), in which an excess of alkaline hydrazine solution has already been placed. An instantaneous and quantitative reaction occurs, according to the reaction



If reaction (1) went completely from left to right, as does reaction (2), 1 mol of  $\text{SO}_4$  would yield 3 mols of  $\text{N}_2$ , and 1 combining equivalent of  $\text{SO}_4$  would yield 1.5 mols of  $\text{N}_2$ . Then  $\text{SO}_4$  could be calculated from  $\text{N}_2$  simply as:

$$(3) \text{ (Stoichiometric.) } \text{Equivalents } \text{SO}_4 = \frac{\text{mols } \text{N}_2}{1.5} = 0.667 \times \text{mols } \text{N}_2$$

However, reaction (1) does not go completely from left to right. The difference in solubility between  $\text{BaSO}_4$  and  $\text{Ba}(\text{IO}_3)_2$  is not great enough quite to cause a complete reaction. Equilibrium is reached when there is still a significant part of the  $\text{SO}_4$  in solution not replaced by  $\text{IO}_3$ . The actual relationships are given by empirical Equation 4 with a somewhat larger factor for  $\text{N}_2$ , and an added term.

$$(4) \quad \text{(Actual.) } \text{M.-eq. } \text{SO}_4 \text{ per liter} = 0.724 \times (\text{mm } \text{N}_2 \text{ yielded per liter}) + 1.123$$

This empirical linear equation is exact, as will be shown, when the reaction between  $\text{Ba}(\text{IO}_3)_2$  and alkali sulfate is carried out within the usual range of room temperature, with  $\text{SO}_4$  concentration between 3 and 15 milli-equivalents per liter, pH between 3 and 7, and in the absence of salts other than sulfates.

<sup>1</sup> This reaction has been used by Riegler (6) for gasometric determination of iodate.

*Description of Method for Total Base in Serum.*

*Reagents.*

*Sulfuric acid, concentrated.*

*Nitric acid, concentrated.*

*4 N Ammonium Hydroxide.*—Dilute 1 volume of concentrated ammonia solution 4 fold.

*1 N sulfuric acid.*

*Ferric Ammonium Sulfate.*—3.18 gm. of crystalline ferric alum in 100 cc. of water.

*0.1 N ammonium hydroxide.*

*Phenol Red Indicator Solution.*—0.1 gm. of the dry powder is ground in a mortar with 5.7 cc. of 0.05 N sodium hydroxide. When solution is complete, dilute to 250 cc.

*Acetic acid solution, 0.2 per cent.*

*Barium iodate, pulverized* (we have used Kahlbaum's).

*Hydrazine Solution.*—Mix equal volumes of saturated aqueous solution of hydrazine sulfate and 40 per cent sodium hydroxide. The hydroxide solution was prepared from Merck's reagent sodium hydroxide made from sodium.

*Procedure.*

*Ashing with  $H_2SO_4$  and  $HNO_3$ .*—Measure 1 or 0.5 cc. of serum into a large Pyrex or silica test-tube (25 × 200 mm.) which has been previously calibrated at 25 cc. The tube is constricted and the calibration mark is made in the constricted area. Add 0.5 cc. of concentrated sulfuric acid, 1 cc. of concentrated nitric acid, and a glass bead. Digest until a dark brown color appears, remove the tube from the flame, and while hot add more nitric acid, a drop at a time, and digest again. Repeat this process two or three times, until the liquid is perfectly clear and all brown fumes have been driven off.

*Removal of Phosphoric Acid.*—Cool, dilute to about 10 cc. with distilled water, add 1 drop of phenol red. The phosphates are removed as described by Stadie and Ross (3). Neutralize with 4 N ammonium hydroxide, then render just acid with a few drops of normal sulfuric acid. Add 1 cc. of ferric ammonium sulfate solution, and 0.1 N ammonium hydroxide till full red



alkaline color of indicator develops. Dilute to 25 cc. mark, and filter through a dry ash-free filter paper.

*Ignition of Sulfates.*—Transfer 20 cc. of the filtrate to a silica or Pyrex dish, add 1 drop of concentrated sulfuric acid, and evaporate to dryness on the steam bath. When as dry as possible, heat *slowly* on an electric stove, bringing the stove finally to full heat until all of the sulfuric acid is driven off. Then heat 15 minutes in the full flame of a triple Bunsen burner. To the residue in the dish add exactly 10 cc. of the acetic acid solution.

*Reaction of Sulfates with Barium Iodate.*—When the residue has gone into solution, pour into a Pyrex test-tube  $15 \times 2$  cm., add 0.25 gm. of barium iodate, stopper tightly, and shake *vigorously* for 1 hour in a shaking machine.

*Gasometric Determination of Dissolved Iodate.*—Filter through a dry ash-free filter paper and estimate the iodate in the filtrate with the manometric gas apparatus (4, 5). The apparatus, before the first analysis, is rendered gas-free by shaking 1 minute with about 2 cc. of the hydrazine solution mixed with 2 cc. of distilled water. The gas formed and the solution are expelled from the apparatus, which is now ready for the analysis. Exactly 2 cc. of the hydrazine solution are run into the apparatus through a mercury seal as previously described (5). Through the same mercury seal, there are then added exactly 2 cc. of the filtrate to be analyzed. The cock of the gas apparatus is sealed with a drop of mercury in the usual way, and the chamber is evacuated until the mercury in it falls to the 50 cc. mark. The chamber is then shaken 1 to 1.5 minutes. The gas volume is brought to 2 cc.; the pressure on the manometer and the temperature are recorded. The manometer reading is  $p_1$  in the calculation.

*Blank.*—Run through the whole procedure using all reagents and processes described, omitting the serum. The manometer reading in the blank is  $p_0$  in the calculation. Blanks must be run with each group of determinations made in the gas apparatus. The blank corrects for impurities in the reagents, for the amount of air dissolved in the hydrazine and iodate solutions (this air is extracted and measured with the  $N_2$  from the iodate), and also for the slight amount of barium iodate which dissolves, by virtue of its slight but measurable solubility, independently of the reaction with the alkali sulfate.

### Calculations.

The millimols of  $N_2$  evolved per liter of filtrate from the equilibrated iodate-sulfate mixture are calculated by means of a factor derived from Table III of Van Slyke and Neill. If we call  $f$  the factor from that table, which gives millimols of  $N_2$  per liter of solution analyzed when the pressure of  $N_2$  from a 1 cc. sample is measured at 2 cc. volume ( $a = 2$ ), then we have

$$(5) \quad [N_2] = \text{mm } N_2 \text{ from 1 liter filtrate} \quad \frac{P f}{\text{cc. sample}}$$

where  $P$  is the pressure difference,  $p_1 - p_0$ , observed in the analysis, and "cc. sample" is the cc. of filtrate (2 cc.) pipetted into the apparatus. We substitute this value of  $[N_2]$  into Equation 4, in order to obtain directly the relationship between the observed manometer pressure and the  $SO_4$  content of the original solution. We thus obtain for the special case in which the sample is 2 cc.,

$$(6) \quad \begin{aligned} \text{M.-eq. } SO_4 \text{ per liter} &= \frac{0.724 f P}{2} + 1.123 \\ &= 0.362 f P + 1.123 \end{aligned}$$

1 liter of the final sulfate solution obtained by the procedure for total base determination described above, when 1 cc. of serum is used, is equivalent to 80 cc. of serum. Hence, for this special case, we multiply the observed  $SO_4$  milli-equivalent concentration of the analyzed final solution by  $\frac{1000}{80}$  to obtain the milli-equivalents of total base (combined with the  $SO_4$ ) per liter of serum. Multiplying the right hand member of Equation 6 accordingly by  $\frac{1000}{80}$  we obtain:

$$(7) \quad \text{M.-eq. base per liter serum} = 4.525 fP + 14.0$$

If only 0.5 cc. of serum is used as the original sample, the calculation becomes

$$(8) \quad \text{M.-eq. base per liter serum} = 2(4.525 fP + 14.0)$$

From inspection of Equations 4 and 7 it is evident that the calculations of  $SO_4$  and total base from observed nitrogen pressures

may be facilitated by the use of a table with values of  $0.724 f$  and  $4.525 f$  respectively. These values are accordingly given in Table I.

TABLE I.

Values of  $0.724 f$  and  $4.525 f$  for Use in Calculation of Equations 4, 7, and 8.

Temperature.	$f$	$0.724 f$	$4.525 f$
°C.			
15	0.1113	0.0806	0.504
16	09	03	02
17	05	00	00
18	01	0.0797	0.498
19	0.1097	94	96
20	93	91	95
21	89	88	93
22	85	86	91
23	81	83	89
24	77	80	87
25	74	78	86
26	70	75	84
27	67	72	83
28	63	70	81
29	59	67	79
30	55	64	77
31	52	62	76
32	48	59	74
33	44	56	72
34	41	54	71

$f$  = factor from Table III of Van Slyke and Neill (4) for calculating mols  $N_2$  per liter solution when the gas is extracted from a 1 cc. sample and the pressure is measured with the gas at 2 cc. volume.

Equation 4 is (*m.-eq.  $SO_4$  per liter solution analyzed*) =  $\frac{0.724 f P}{\text{cc. sample}} + 1.123$ .

Equation 7 is (*m.-eq. base per liter serum*) =  $4.525 f P + 14.0$ .

$P = p_1 - p_0$  observed in analysis.

*Example.*—In determination of total base in serum as above described with a sample of 1 cc. of serum, the  $(p_1 - p_0) = P$  reading was 285 mm. at 20°. With the factor in Table I by Equation 7 we therefore calculate

$$\begin{aligned}
 \text{M.-eq. base per liter serum} &= 0.495 \times 285 + 14.0 \\
 &= 141.1 + 14.0 \\
 &= 155.1
 \end{aligned}$$

## EXPERIMENTAL.

*Gasometric Estimation of Iodates with Hydrazine.*

2 cc. of a standard 0.012 M solution of potassium iodate were shaken 1 minute in the gas apparatus with 2 cc. of the hydrazine solution. The amount of nitrogen gas liberated was found to be theoretical according to reaction (2), already given in the introduction. The molal relationship of iodate to  $N_2$  yield is found within the limit of error, to be that calculated,  $KIO_3:N_2 = 2:3$ . The results are shown in Table II. The 12 millimolar solution of potassium iodate liberated 18 millimols of nitrogen.

TABLE II.  
*Gasometric Estimation of Iodates with Hydrazine.*

Potassium iodate per liter.	P	Temperature.	$N_2$ liberated per liter.	Theoretical $N_2$ per liter.
mm	mm.	°C.	mm	mm
12.00	328.8	20.3	17.95	18.00
	329.6	21.1	17.95	
	333.4	21.9	18.09	
	330.2	21.4	17.94	
	330.2	21.5	17.94	
	334.0	22.5	18.09	

*Reaction between Barium Iodate and Alkali Sulfates.*

Potassium sulfate was recrystallized and fused. A standard 0.1 N solution, containing 8.713 gm. per liter, was prepared for the following experiments.

*Optimum Proportion of Barium Iodate for the Reaction with Dilute Sulfate Solutions.*—In order to ascertain the desirable amount of barium iodate for sulfate solutions of the concentration encountered in blood analyses a portion of the stock 0.1 N  $K_2SO_4$  solution was diluted to 0.01 N. 10 cc. portions were placed in Pyrex test-tubes with varying amounts of Kahlbaum's potassium iodate, and the mixtures were shaken vigorously for 1 hour in a shaking machine. The solutions were then filtered through dry papers, and the iodate in the filtrates was estimated by transferring 2 cc. portions to the gas apparatus with hydrazine, as above described. In accordance with the results in Table III, 0.25 gm. of iodate was chosen as the amount for routine use.

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TABLE III.

*Amount of Barium Iodate Necessary for Gasometric Estimation of Sulfate.*

Concentration $\text{SO}_4$ per liter.	$\text{Ba}(\text{IO}_3)_2$	Nitrogen liberated per liter.*
<i>m.-eq.</i>	<i>gm.</i>	<i>mm</i>
15	0.05	19.02
	0.05	18.96
	0.10	19.12
	0.10	19.06
	0.25	19.14
	0.25	19.18
	0.35	19.15
	0.35	19.18

\* Theoretical for complete replacement of  $\text{SO}_4$  by  $\text{IO}_3$  is 22.5 millimols.

TABLE IV.

*Determination of Time of Shaking Necessary to Bring about Equilibrium in Reaction between Dissolved Sulfates and Solid Barium Iodate.*

Concentration $\text{SO}_4$ per liter.	Time of shaking.	Nitrogen liberated per liter.*
<i>m.-eq.</i>	<i>hrs.</i>	<i>mm</i>
15	0.5	19.04
	0.5	19.05
	1	19.15
	1	19.18
	2	19.19
	2	19.20

\* Theoretical for complete replacement of  $\text{SO}_4$  by  $\text{IO}_3$  is 22.5 millimols.

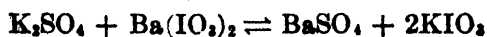
TABLE V.

*Effect of the pH of the Solution on Equilibrium Attained in Reaction between Dissolved Sulfate and Solid Barium Iodate.*

Concentration $\text{SO}_4$ per liter.	pH of solution during reaction of $\text{K}_2\text{SO}_4$ and $\text{Ba}(\text{IO}_3)_2$ .	Nitrogen liberated per liter.	
		Found.	Calculated for complete reaction of $\text{Ba}(\text{IO}_3)_2$ and $\text{K}_2\text{SO}_4$ .
<i>m.-eq.</i>		<i>mm</i>	<i>mm</i>
15	2.0	19.42	22.50
	2.5	19.32	
	3.0	19.20	
	4.0	19.15	
	7.0	19.13	
	7.3	20.22	
	8.3	21.64	

The length of time required to bring about equilibrium in the reaction between alkali sulfate solution and barium iodate was determined by repetition of the experiment just described, except that the amount of barium iodate, 0.25 gm., was constant in each tube, while the time of shaking was varied. The results given in Table IV, show, within the limits of experimental error, that the equilibrium is complete in 1 hour.

In order to determine the effect of reaction on the equilibrium



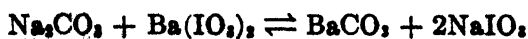
an experiment was performed as just described, with the amount of barium iodate constant in each tube, while the reaction was varied by addition of varying slight amounts of acetic acid, hydrochloric acid, or sodium hydroxide. The tubes were shaken 1 hour. The data in Table V show that constant results are

TABLE VI.

*Effect of Presence of Other Salts in Solution during Establishment of Equilibrium Reaction.*

Concentration potassium sulfate per liter of solution.	Concentration sodium acetate per liter of solution.	Nitrogen liberated per liter of solution.
m.-eq.	mm	mm
12	0	15.03
12	50	14.34
6	0	6.56
6	50	6.07

obtained between pH 3 and 7. This fact makes it possible to carry out the reaction in solutions of sulfates acidified with acetic acid; so that the presence of carbonates and any interference by the reaction



are avoided.

The curve representing the relationship between the initial concentration of soluble sulfate and the final concentration of iodate dissolved by the reaction with barium iodate was estimated by shaking varying concentrations of potassium sulfate solution in 10 cc. portions for 1 hour at room temperature, between 20 and 25°C.,

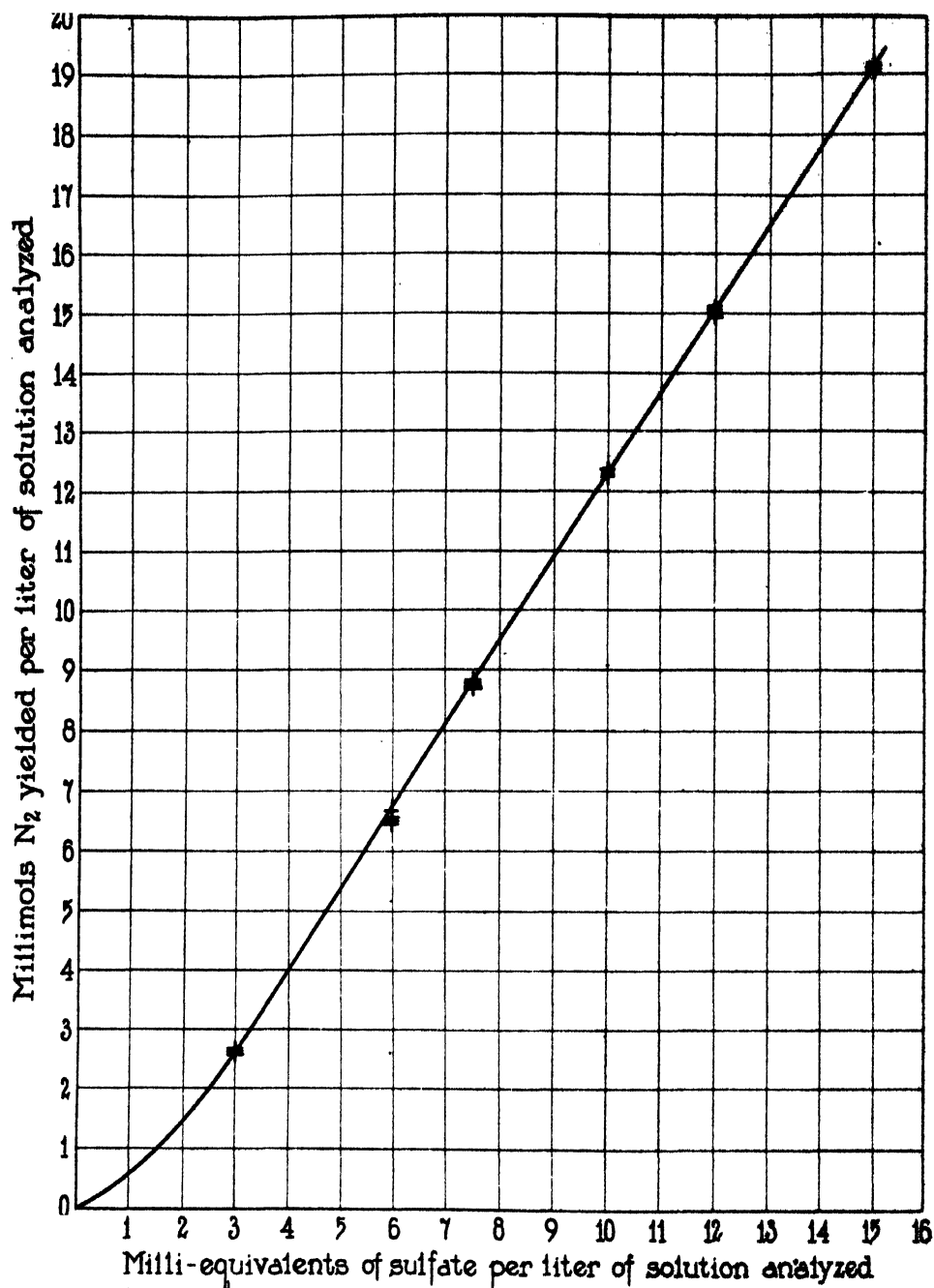


FIG. 1. Relationship between  $\text{SO}_4$  content of solution shaken with barium iodate and the nitrogen gas liberated from the filtrate by reaction of the dissolved iodate with hydrazine. Each cross mark represents the result of a separate equilibration of barium iodate and potassium sulfate.

with 0.25 gm. portions of barium iodate. The mixture was filtered, and 2 cc. portions were analyzed in the gas apparatus with 2 cc. of the hydrazine solution. The results of four series of experiments are shown in Fig. 1. The maximal difference in the estimation of duplicate points on the curve was 0.84 per cent. The equation representing the straight portion of the curve has already been given (Equation 4).

*Effect of Presence of Other Salts in the Solution during Equilibration of Barium Iodate and Alkali Sulfate.*—Solutions containing respectively 0.12 and 0.06 milli-equivalents of potassium sulfate,

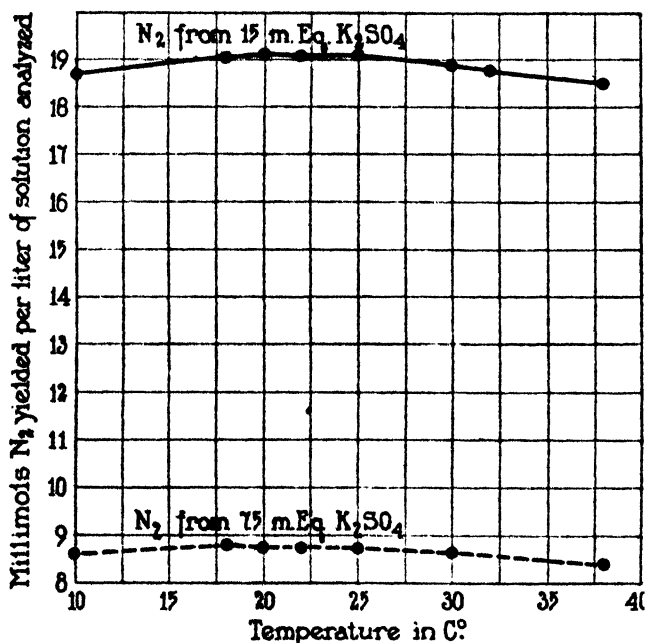


FIG. 2. The effect of temperature on the reaction of barium iodate and alkali sulfate.

and in addition sodium acetate in 50 millimolar concentration, were shaken with barium iodate in the usual manner. The results in Table VI show that in the presence of sodium acetate the end-point of the reaction  $\text{K}_2\text{SO}_3 + \text{Ba}(\text{IO}_3)_2 \rightleftharpoons \text{BaSO}_4 + 2 \text{KIO}_3$  was shifted to the left, presumably because of the effect of the added salt (acetate) on the solubilities of barium iodate and barium sulfate. The amount of iodate in the final solution was diminished by the presence of sodium acetate during the shaking.



## 670 Gasometric Iodate, Sulfate, and Base

The effect of temperature on the equilibrium point of the reaction  $K_2SO_4 + Ba(IO_3)_2 \rightleftharpoons 2KIO_3 + BaSO_4$  was determined by shaking

TABLE VII.

*Gasometric Estimation of Base in Standard Potassium Sulfate Solution by the Method for Total Base in Serum.*

Amount of solution used for estimation.	P	Temperature.	4.525 f	Base found per liter.	Theoretical amount base per liter.
cc.	mm.	°C.		m.-eq.	m.-eq.
1	277.6	20.6	0.494	151.1	
	278.5	21.0	0.493	151.3	
	278.8	21.1	0.493	151.4	
	278.6	21.2	0.493	151.3	
	279.2	21.3	0.493	151.6	
Average.....				151.3	150.0
0.5	126.2	24.7	0.486	150.7	
	125.4	24.7	0.486	149.9	
	123.7	24.7	0.486	148.2	
	125.2	24.7	0.486	149.7	
	124.4	24.7	0.486	148.9	
Average.....				149.7	150.0

TABLE VIII.

*Recovery of Base Added to a Solution of Serum Proteins.*

	P	Temperature.	4.525 f	Base found per liter of solution.	Theoretical base added per liter of solution.
	mm.	°C.		m.-eq.	m.-eq.
Dialyzed protein solution.	0	27.6		0	
	0			0	
	0			0	
Dialyzed protein solution and NaCl.	286.8	29.3	0.479	151.4	
	280.8	25.6	0.485	150.2	
	280.2	25.6	0.485	149.9	
Average.....				150.5	150.0

10 cc. portions of sulfate solution with 0.25 gm. portions of barium iodate for 1 hour at varying temperatures and determining

the dissolved iodate gasometrically in the usual way. The blanks were, of course, shaken at the same temperatures as the sulfate solutions. The results, given in Fig. 2, show a practically negligible effect between 18 and 25°. Consequently room temperature variations within ordinary limits can be disregarded in the use of the method.

*Recovery of Total Base from Standard Sulfate Solutions  
Treated by Procedure Used for Serum.*

Standard potassium sulfate solutions were put through the whole process, described above, for estimating total base in serum. In order to use the same amounts found in serum, 1 cc. and 0.5 cc. portions of a solution containing 150 milli-equivalents of  $K_2SO_4$  per liter were used. The results are shown in Table VII.

*Recovery of Base Added to a Solution of Serum Proteins.*

A solution of serum proteins was dialyzed until free of salts. The protein content of the solution was 24.94 gm. per liter. The solution was digested with sulfuric and nitric acids and analyzed for total base both before and after the addition of 150 millimols of sodium chloride per liter. The results in Table VIII show a theoretical recovery.

*Comparative Estimation of Total Base in Serum by Gasometric  
and Titration Methods.*

Three methods were compared: the gasometric method described above, the benzidine method of Stadie and Ross (3), and a benzidine method which was in routine use in this laboratory before the present gasometric method was available. For all three methods the conversion of base into neutral sulfates was done according to the method described above, by ashing with nitric and sulfuric acids, and removing phosphates with ferric sulfate and ammonia. The benzidine method used in this laboratory is the following.

*Benzidine Method.*

*Reagents.*

*Benzidine.*—4 gm. dissolved in 50 cc. of 1 N hydrochloric acid and diluted to 250 cc. with distilled water. If a brown residue forms, the solution should be filtered through ash-free filter paper before using.

*Acetone.*—95 per cent.

## 672 Gasometric Iodate, Sulfate, and Base

*Phenolphthalein Indicator Solution.*—1 per cent in 50 per cent alcohol. 0.02 N sodium hydroxide, which is standardized by titration with phenolphthalein, the first faint permanent pink as the end-point being used.

*Procedure.*—To the residue of neutral sulfates in the dish add 10 cc. of distilled water and 1 drop of phenolphthalein, and titrate with 0.02 N sodium hydroxide. If the free sulfuric acid has been properly driven off, this titration is only 0.02 or 0.03 cc., which is the amount necessary to change the color of the indicator in that amount of distilled water, so this titration can be omitted after the technique of the ignition has become assured. Add 2 cc. of the benzidine solution and 4 cc. of 95 per cent acetone. Let stand about 10 minutes and filter through a 5.5 cm. ash-free filter paper in a 3.5 cm. funnel. Wash with 15 cc. of 95 per cent acetone, using four or five 1 cc. portions to wash the dish, the remainder to wash the precipitate on the filter paper. Loosen the filter paper from the funnel, let stand a few moments until some of the acetone has evaporated and the paper just begins to dry. Transfer the paper to a 50 cc. centrifuge tube, add 10 cc. of distilled water, and heat in a water bath until the odor of acetone disappears. Add 1 drop of phenolphthalein and titrate hot with 0.02 N sodium hydroxide to the first faint permanent pink, with care to observe that all particles of the benzidine sulfate precipitate have dissolved. If they have not, reheat and titrate again. Use a burette graduated into divisions of not more than 0.05 cc., so that readings can be estimated to 0.01 cc.

*Blank.*—Run through the whole procedure using all reagents and processes described, omitting the serum. The blank is usually found to be between 0.10 and 0.30 cc. of 0.02 N sodium hydroxide for the amounts of reagents here described.

*Calculation.*—(Cc. 0.02 N NaOH used in titration — cc. 0.02 N NaOH used in blank)  $\times$  25 = m.-eq. base per liter of serum.

The results, in Table IX, by the three methods show good agreement, the differences between the methods falling within the limits of experimental error of any one of them. Each of the methods showed about the same experimental error, 0.8 per cent of the amount of base determined.

*Estimation of Total Base in Serum after Precipitation of the Proteins with Trichloroacetic Acid.*—The same serum used in the previous experiment was treated with 9 volumes of 10 per cent trichloroacetic acid. After 20 minutes the proteins were filtered off and 10 cc. of the filtrate, equivalent to 1 cc. of serum, were put through the gasometric procedure described above for total base. The results in Table X show a 4 per cent increase in the figures obtained from the trichloroacetic acid filtrate over those from the direct estimation on wet-ashed serum. Repetitions of the experiment gave similar results.

Analyses of solutions containing serum proteins and known amounts of base were made in order to determine whether the trichloroacetic acid precipitation of the proteins or their destruction by nitric and sulfuric acid gives a residue yielding the correct amount of base. To prepare a salt-free solution of serum proteins, horse serum was subjected to pressure dialysis in collodion sacs against water saturated with carbon dioxide. The dialyzed serum was analyzed for base both before and after sodium chloride

TABLE IX.  
*Comparison of Methods for Estimation of Total Base in Serum*

Method.	Amount of serum ashed.	Amount of serum represented in solution used for final analyses.	Base found per liter of serum.	Average deviation from mean.
	cc.	cc.	m.-eq.	per cent of mean
Gasometric.	1.0	0.16	148.2	0.81
	0.5	0.08	146.6	0.51
Benzidine.	1.0	0.80	147.6	0.81
Stadie and Ross (3).	1.0	0.80	147.5	0.88

TABLE X.  
*Estimation of Total Base in Trichloroacetic Acid Filtrate Compared with Direct Estimation in Ashed Serum.*

Method.	Base found per liter of serum.
	m.-eq.
Direct.....	148.2
Trichloroacetic acid filtrate.....	154.1

had been added. A solution of purified serum proteins was put through the same procedure.

Table XI shows a theoretical recovery of base added to dialyzed serum or serum protein solution when the base is determined after destruction of the proteins by the wet-ashing method. But the trichloroacetic acid filtrate yielded results 4 per cent higher than the amount of sodium added.

Control analyses in which the salt solution was put through

the trichloroacetic acid treatment yielded theoretical recoveries of base, as shown by Table XII.

Apparently, when the proteins are coagulated with trichloroacetic acid, the coagulum contains less fixed base per cc. than the supernatant liquid, so that analysis of aliquots of the super-

TABLE XI.  
*Recovery of Base Added to Dialysed Serum and Serum Proteins.*

Solution.	Proteins destroyed by $\text{HNO}_3\text{-H}_2\text{SO}_4$ digestion. Base found per liter.			Proteins precipitated with trichloroacetic acid. Base found per liter.		
	Dialysed solution.	Dialysed solution + NaCl.	Base added per liter.	Dialysed solution.	Dialysed solution + NaCl.	Base added per liter.
	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.
Serum.		149.3 152.0 152.0			154.4 156.2 158.1	
Average.....	0	151.1	150.0	1.6	156.2	150.0
Serum proteins.		149.4 151.2 152.5			158.1 156.2 156.8	
Average.....	0	151.0	150.0	0	157.0	150.0

TABLE XII.  
*Recovery of Base in a Pure Salt Solution in Presence of Trichloroacetic Acid.*

Base found per liter of solution.	Base added as NaCl per liter of solution.
m.-eq.	m.-eq.
151.2	
150.0	
149.6	
Average..... 150.3	150.0

natant solution yields high results for total base estimated on the assumption that the latter is evenly distributed through coagulum and solution. The total base analyses of Stadie and Ross (3) also show higher results with the trichloroacetic acid filtrate than with the residue obtained by the wet-ashing process.

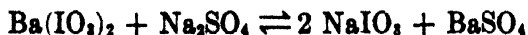
## SUMMARY.

A rapid and precise gasometric micro determination of iodates, in the manometric apparatus of Van Slyke and Neill, by the reaction



is described.

Sulfates are determined by gasometric estimation of the iodate dissolved when the sulfate solution is equilibrated with excess solid barium iodate. The reaction



does not go completely from left to right, but reaches an exactly reproducible equilibrium, fixed by the relative solubilities of barium sulfate and iodate.

For determination of total base in serum, the latter is ashed with nitric and sulfuric acids, and the bases are converted into sulfates as described by Stadie and Ross (3). The  $\text{SO}_4$  is then determined by the above procedure. The base from 0.16 cc. of serum suffices for a determination, with an average error of 0.8 per cent of the amount determined.

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# AN ELECTROLYTIC METHOD FOR THE DETERMINATION OF SODIUM PLUS POTASSIUM.

By JAMES L. STODDARD.

(From the Chemical Laboratory of the Massachusetts General Hospital, Boston.)

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The method described in this paper is designed for the determination of the sum of sodium and potassium, and thus of sodium by difference, if potassium is determined.

The material is first ashed and dissolved. Magnesium and calcium are then precipitated. Part of the remaining solution is electrolyzed in a simple apparatus, consisting mainly of one test-tube fused inside another, with mercury in each tube, and a passage through the wall of the inner tube (Fig. 1). Under an E.M.F. of 110 volts the negative ions migrate to the mercury in the outer tube. The positive ions migrate to the mercury in the inner tube, where they form an amalgam, which is then removed and titrated.

Results check within 2 per cent when the quantity of sodium plus potassium is equal to at least one-fourth that contained in 1 cc. of plasma.

The method was designed principally for use with the new and accurate method for the determination of potassium of C. H. Fiske. Unfortunately up to the moment of writing only a preliminary note on this method has been published.

*Development of the Method.*—It was found early in the investigation that a voltage of 110 is necessary to give results in a reasonably short time. Even with this voltage, however, it is necessary to reduce the maximum distance of travel of ions as much as possible. A mechanism by which the amalgam formed could be shut off from the remainder of the solution and then titrated seemed to involve the use of a stop-cock or pinch-cock; but such devices increased the distance to be traveled to an impractical



figure. The concentric apparatus of Fig. 1 was then invented. Mercury in the inner tube acts as cathode, mercury in the outer tube as anode. The solution to be analyzed floats on the mercury in both compartments and connection is had through the holes in the inner tube. Connection is broken by tilting the apparatus so that the level of liquid drops below the connecting holes. Experiments showed that in the upper four-fifths, at least, of the

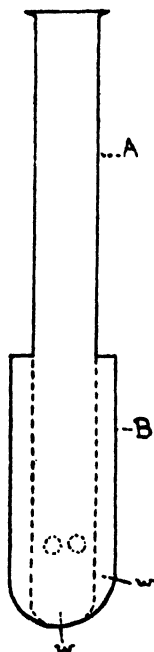


FIG. 1.

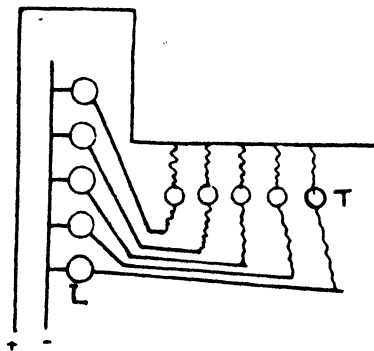


FIG. 2.

FIG. 1. *A* represents the inner tube, *B* the outer tube, and *w* one of the platinum wires.

FIG. 2. *T* indicates the electrical apparatus, *L* the incandescent lamp.

liquid there is nothing titratable at the end of the amalgam formation, so that the very slight mixing possible by uneven manipulation of the apparatus would cause no error. The mercury and liquid in the inner tube are then run out into another tube and titrated. (Details are given later.)

*Description of Apparatus.*—A small Pyrex test-tube, of about 12 mm. internal diameter and 13 cm. length, with fairly thin wall, is fused at the bottom inside a short Pyrex test-tube, internal diameter about 20 mm., length 6.5 cm. There should be about

a 3 mm. space between the tubes (Fig. 1). The inner tube has two holes in one side, about 19 mm. from the bottom and about 3 to 4 mm. in diameter, placed horizontally. One platinum wire is fused into the bottom of the inner tube where the latter is fused to the outer tube, and another is fused into the outer tube about 0.9 cm. from the bottom.

*Directions for Making the Apparatus.*—Cut off the lower 6.5 cm. of the 20 mm. internal diameter Pyrex tube as follows: With a new or very sharp file make a circular mark deeply at the place to be cut, and completely around the tube; then heat one point on this line with a very small fine flame of the blast lamp, and immediately touch the heated place with a drop of water; then pull apart the two sections of the tube. Fuse the platinum wire into the side of the tube, and fasten it in firmly by melting a fine sliver of Pyrex glass about it as it emerges from the tube. Then, with a pair of snaps or forceps, grasp the inner end of the wire so as to break off some of the glass and allow the possibility of electrical contact. Blow the two holes in the smaller tube, place it in the larger one, and fuse it in at the bottom. Insert a platinum wire into the smaller tube through the fused place at the bottom, and fasten it as described above. Heat the whole apparatus in a smoky flame until well covered with soot, then wrap in a cloth until cool. Break off the glass on the inner part of the platinum wire in the inner tube as described, or by carefully pushing the sharp edge of a triangular file down along the side of it.

*Description of Electrical Connections.*—Each tube is connected in series with a 25 watt 250 volt lamp on a 110 volt direct current (see Fig. 2). In case such a circuit is not at hand, radio B batteries to make this voltage would undoubtedly work equally well. The amount of current used is very small. The platinum wire of the tube is conveniently connected with the circuit wires with fine covered copper wire, such as No. 26 gauge. Since the usual covering is too thin to insulate perfectly against 110 volts, it is important not to allow the wires to remain in contact with each other. The outer wire of each tube is connected to the positive pole, and the inner wire with one terminal of a lamp, the other terminal of which is connected to the negative pole. A large number of tubes can be connected to one electrical outlet.

The clamps holding the tubes are conveniently attached to a horizontal rod—an iron stand on its side, for instance.

*Directions for the Method.*—Ash the material by the wet method, as follows (these details are taken from Fiske's total base method): To 1 cc. of plasma or urine (or what other amount may be advisable, as discussed later) in a non-protein nitrogen tube (a Pyrex test-tube about 20 by 200 mm.) add one or two pebbles, 1 cc. of 4 N sulfuric acid, and 0.5 cc. of nitric acid. Boil until dense white fumes fill the tube, then heat for 3 or 4 minutes more. If the solution is not clear, let it cool a little, add 0.5 cc. more of nitric acid, and boil again. Usually urine becomes clear after the first heating and plasma after the second, but other material may require much more ashing.

Let cool, add about 2 cc. of distilled water, mix, and transfer to a platinum dish. Rinse four times down the sides of the tube with about 1 or 2 cc. of water each time. Evaporate the water on the steam bath. Place the platinum dish in a Pyrex beaker on a tin or thin iron plate which is placed on wire gauze over a Meeker burner. Heat very slowly, gradually raising the flame until the plate shows a dull red heat, and keep this heat until fumes cease to come out of the crucible. Then place the crucible on a triangle and heat all parts to a dull red heat for a moment with a micro burner. Four or five platinum crucibles can be heated at once on a tin plate in this manner.

Let cool. Add exactly 2 cc. of approximately 0.012 HCl (dilute the concentrated HCl 1:1000). Warm slightly over a small flame of a micro burner, then stir with a rubber-tipped glass rod, rubbing all the interior.<sup>1</sup> Add exactly 1 cc. of precipitating solution (1.0 per cent of very pure  $(\text{NH}_4)_2\text{HPO}_4$  in 10 per cent of concentrated  $\text{NH}_4\text{OH}$  c.p. (sp. gr. 0.9)). Mix thoroughly. Pour back and forth twice into a Pyrex centrifuge tube with the aid of the glass rod, mixing again in the crucible each time, and rinsing the inner surface, rubbing it with the policeman. Let stand in the centrifuge tube, tightly stoppered, for  $\frac{1}{2}$  to 4 hours. Centrifuge for 15 minutes.

Remove 1 or 2 cc., keeping the finger over the end of the pipette as it is introduced into the solution in order to avoid drawing up floating crystals, and wipe off the end of the pipette carefully after removing. Put this measured amount into a 30 cc. Pyrex

<sup>1</sup> There will usually be present an undissolved precipitate of calcium, magnesium, and phosphate, of course.

beaker and evaporate on the steam bath. When dry add about 0.6 cc. of approximately 0.15 N HCl (dilute the concentrated 1:80) if 1 cc. of solution is taken, or 1.2 cc. if 2 cc. are used. Evaporate again. Put in an air oven at about 115° for 10 minutes on a piece of asbestos, or a pad of paper, in order not to heat the bottom quickly, so that it may dry thoroughly without spattering. If this step is omitted, the heating done next must be very gradual at first.

Place on a tin plate on gauze over a Meeker burner and heat gradually, at first with the flame just touching the gauze, until fumes come off, then raise the flame until the tin plate shows a very dull red heat directly over the flame, and keep this heat till fumes of  $\text{NH}_4\text{Cl}$  cease to evolve. Then put the burner directly under each beaker in turn for about 60 seconds. Remove each beaker with forceps and heat the sides to remove any condensed  $\text{NH}_4\text{Cl}$ . Let cool.

Add about 1 cc. of distilled water and approximately 0.15 cc. of the 0.15 N HCl. Heat over a micro burner until it steams, stirring gently and cautiously with a rubber-tipped glass rod. Let it stand a minute, then heat again, with stirring.

From a separatory funnel with fine tip attached run pure, and tested, mercury (test by titration as described later) into the inner tube of one of the pieces of apparatus to a level a little below the holes, then fill the outer tube to the same level. Clamp vertically, by the outer tube. Attach the outer tube wire to the positive electrical connection, and the inner tube wire to the negative.

With the aid of the glass rod, transfer the solution in the beaker to the inner tube, then rinse the inside of the beaker four times with about 0.5 cc. of distilled water each time. Then rinse the inside of the tube with 0.5 cc. Do not get the total volume in the tube over about 4 or 5 cc.

Leave the tube with the current on for 1 to 2 hours.

Empty the amalgam which has formed in the inner tube into a titration tube consisting of a Pyrex test-tube about 6 inches long by  $\frac{3}{4}$  inch internal diameter narrowed somewhat near the top. In order to do this without letting any of the outer liquid or mercury get into the titration tube it is best to tie a narrow strip of cloth about the inner tube about 2 inches from the end.

Place the titration tube in a beaker, with another beaker of the same height next to it on the right to catch the outer liquids. Loosen the clamp holding the apparatus, then carefully, without tipping it, or moving it suddenly (in order to avoid any movement of liquid or mercury through the holes in the inner tube) take it out of the clamp. Then smoothly and rapidly tip it toward the side opposite the holes to an angle of about  $45^\circ$  so as to bring the surface of the liquid below the holes, thus shutting off connection between the two compartments of the tube. Then place the end of the inner tube over the edge of the titration tube and empty the inner tube into it taking care not to let the mercury run over the further edge of the titration tube. It is well to tip the titration tube further toward the apparatus at this point. Then drain the inner tube with the aid of a fine glass rod (hooked at the top so that it can hang on the titration tube). Hold the apparatus vertically, rinse down the sides of the inner tube with about 0.5 cc. of distilled water, empty this into the titration tube, then repeat the rinsings twice. The wires should have been connected and the current on up to this point.

*Titration.*—Add 1 cc. of 0.1 N HCl and 4 cc. of distilled water, bring to boiling over the micro burner, with constant stirring by a circular motion, then clamp at an angle of about  $60^\circ$  and boil for 3 minutes. Take out of the clamp, add phenol red, hold in a test-tube holder, and titrate with 0.02 N NaOH from a micro burette with a fine tip, keeping the solution gently boiling.<sup>2</sup> The alkali should be standardized by a similar titration, and in a similar volume of solution, and with a preliminary boiling of 3 minutes to remove  $\text{CO}_2$ . The alkali should be kept in a Pyrex or paraffined bottle and guarded with soda-lime. It should be restandardized often. Of course, if 1 cc. of HCl fails to make the solution acid, another cc. should be added, and the solution boiled for another 3 minutes in order to decompose the remaining amalgam.

*Calculation.*—If 1 cc. of the centrifuged solution is used the

<sup>2</sup> The titration is best done by a strong artificial light placed back of the titrator. Place a white envelope or card along one side of which a strip of yellow paper has been pasted, back of the titration tube, with the white part directly behind it. Just before the end of the titration the color of the solution is close to that of the paper, and by contrast the first trace of pink tint shows out definitely. This is the end-point.

amount of alkali found by titration  $\times 3 \times 1.012$  equals the amount in the original material. If 2 cc. of solution are used, the amount of alkali found  $\times \frac{3}{2} \times 1.012$  equals that in the original amount of material taken.

To clean the apparatus, rinse once with water, then pour concentrated  $\text{HNO}_3$  in the outer tube, and shake until adhering deposits are detached. Rinse three times with water, and three times with distilled water, draining carefully.

If the mercury is pure to begin with, and handled carefully, it can be used again after washing, filtering, and stirring for  $\frac{1}{2}$  hour with a mechanical stirrer in a large beaker with 0.1 N  $\text{HCl}$ , then washing free from acid, drying, and filtering. Each lot should always be tested by placing a few cc. in the titration tube, adding 1 cc. of 0.1 N  $\text{HCl}$ , 8 cc. of distilled water, boiling for 3 minutes, and titrating. If the above method does not purify it, draw a rapid current of air through it for 24 hours, or run it in a fine stream through a tall column of 8 per cent  $\text{HNO}_3$ . Distillation is ordinarily unnecessary.

*Notes on the Method.*—The amount of sodium plus potassium in the amount of material ashed should be between 0.11 and 0.75 cc. of 1.0 N base. Ordinarily 1 cc. of plasma or whole blood will fall between these limits. In the case of urine, the specific gravity would give indication of any unusual concentration; usually 2 cc. would be sufficient. If a low sodium plus potassium content is suspected, or if an extraordinarily high phosphate coexists with a moderately low sodium plus potassium, a larger amount of material should be ashed.

In case a low sodium plus potassium content, near the above limit, is found, and it is impossible or inconvenient to ash a larger specimen, the titrated solution at the end should be tested for ammonia by Nesslerization. If none is present, the determination is not in error. If ammonia is found, the result is too high. Evaporate another cc. of the centrifuged solution, adding before evaporation exactly 1 cc. of 0.05 N  $\text{NaCl}$  (made from the fused salt). Proceed according to the method, but subtract the value this method gives from the  $\text{NaCl}$  solution added; *i.e.*, 0.0494 cc. of 1.0 N.

The explanation of the directions just given is as follows. Un-

less the sodium plus potassium content of the precipitated solution is of the same molar concentration as the phosphate present, not all of the phosphate will be changed later on to  $\text{NaH}_2\text{PO}_4$ , and some ammonium phosphate will remain. It requires a higher degree of heat than is used in this method to drive off ammonia completely from ammonium phosphate, so that not all ammonia would be removed, and an error result. The molar concentration of phosphate from the precipitant is 0.0252. Phosphates in the material, if blood or urine, would not ordinarily bring it to over 0.035, corresponding to an original concentration of sodium plus potassium in the material of 0.105. By adding the sodium chloride solution, all of the phosphate will be enabled to form  $\text{NaH}_2\text{PO}_4$ .

HCl is added after the first evaporation in order to form  $\text{NaH}_2\text{PO}_4$  and  $\text{NH}_4\text{Cl}$ , and thus enable the ammonia to be easily vaporized with the minimum amount of base present. HCl is added in small amount to the final solution of the residue, and it is warmed, in order to insure the solution of any sodium metaphosphate which may have formed during the previous heating of the dry residue.

To ash whole blood, it may be necessary to add a second cc. of sulfuric acid, and to digest it for a long time with repeated additions of small amounts of nitric acid. Charred spots after heating in the platinum dish can be cleared up with a small flame of the micro burner; avoid more than a faint dull red heat for a short time.

It was proved that there is no recognizable difference between using Pyrex beakers and platinum crucibles for the evaporation and heating of the precipitated solution. It is important to avoid having acid fumes or ammonia in the air of the laboratory while the solution is being electrolyzed. It is well to have a slight draft from the worker toward the titration tube while titrating, in order to avoid any possibility of absorbing mercury vapor.

The principal cause of low results seems to be losses by spattering of liquid or solid. Such losses are especially likely if the digested residue in the platinum dish is dried too quickly, or if the later residue dried on the steam bath is heated over the burner without being previously heated in the air oven. In this latter case the dried residue pops at a certain point of the heating, unless it is exceedingly cautiously done.

Blanks can be run without any special trouble on all of the reagents except the ammonium phosphate. If this is done, enough of a standardized sodium (or potassium) chloride solution must be added to enable all of the phosphate to form  $\text{NaH}_2\text{PO}_4$ , and the solution must be evaporated, etc., and heated as in the method.

TABLE I.

Amount.	Time.	Per cent of total found.
cc.	min.	
2	60	99.3
2	60	98.9
2	60	98.4
2	60	98.5
1	69	98.1
1	75	98.9
3	40	99.5
3	40	99.0
3	90	98.5
3	110	98.6
3	170	99.4
1	78	98.14 •
1	80	98.3
2	75	98.8
1	60	98.7
1	60	99.0
1	40	98.5
1	40	98.87
1	45	99.5
1	45	99.15
Total.....20		Average ... 98.80

### *Analysis of Known Solutions.*

*Sodium Chloride.*—In order to standardize the electrical part of the method a sodium chloride solution was made up in 0.1 N concentration from Eimer and Amend T.P. analyzed salt, freshly fused in a platinum crucible, and cooled in a hot air oven, then in a desiccator. The amounts shown in Table I were measured directly into the inner tube of the electrical apparatus, and left for the time indicated. The figures include all results in the



TABLE II.

Cc. 1.0 N base per cc. solution.						Percent of total found, corrected.	Time precipitated.	Solution.
Na	K	Na + K	Mg	Ca	Na + K found, actual.		hrs.	cc.
0.0641	0.0128	0.0769	0.0065	0.0079	0.0738	97.1	2	1
					0.0748	98.5	2	1
					0.0744	98.0	4	1
					0.0764	100.5	4	1
0.1282	0.0128	0.1410	0.0065	0.0079	0.1354	97.2	4	1
					0.1369	98.3	96	1
					0.1390	99.8	4	1
					0.1376	98.8	4	1
0.1282	0.0128	0.1410	0.0021	0.0025	0.1386	99.5	4	1
					0.1364	97.9	4	1
					0.1391	99.8	4	1
0.0641	0.0128	0.0769	0.0065	0.0079	0.0752	99.0	4	1
					0.0732	96.3	4	1
0.100	0.0125	0.1125	0.0100	0.0125	0.1091	97.8	4	1
					0.1089	97.8	4	2
					0.1108	99.6	4	2
					0.1117	100.4	4	1
					0.1115	100.3	4	1
					0.1115	100.3	4	2
					0.1089	97.8	96	1
					0.1121	100.8	96	1
					0.1115	100.3	96	1
					0.1099	98.8	96	1
					0.1115	100.3	3	1
					0.1089	97.8	3	1
					0.1087	97.8	2	1
					0.1067	96.0	2	1
					0.1097	98.7	2	1
0.0892	0.0111	0.1003	0.0137	0.0111	0.0983	99.2	3	1
					0.0973	98.2	3	1

TABLE II—*Concluded.*

Cc. 1.0 N base per cc. solution.						Percent of total found, corrected.	Time precipitated.	Solution.
Na	K	Na + K	Mg	Ca	Na + K found, actual.			
0.08	0.02	0.10	0.012	0.010	0.0996	100.8	hrs. 5	cc. 1
					0.0996	100.8	5	1
					0.0996	100.8	5	1
					0.0965	97.7	2	1
					0.0968	98.0	2	2
					0.0971	98.3	2	1
					0.0991	100.3	2	2
					0.0991	100.3	2	1
					0.0991	100.3	0.75	1
					0.0979	99.1	0.75	2
					0.0985	99.7	0.75	1
					0.0996	100.8	0.75	1
					0.0989	100.1	0.75	2
					0.0997	100.8	0.75	1
					0.0981	99.3	0.75	1
					0.0995	100.7	0.75	1

series arranged in chronological order. On account of the close checking of the results, in spite of variations in time and amount, with a maximum deviation from the average of only 0.7 per cent, it was decided that the 1.2 per cent of undetermined substance was inherent in the method and constant enough to be introduced as a correction. All later results substantiated the correctness of this point of view.

Experiments were then done to find whether additional negative ions would interfere with the determinations. 1 cc. of approximately 0.05 N HCl, of 0.05 N H<sub>2</sub>SO<sub>4</sub>, or of 2.5 per cent trichloroacetic acid failed to move the result out of the range in Table I. 1 cc. of 0.1 N HCl alone in the apparatus gave an absolute blank. 1 cc. of 0.3 N HCl, however, lowered the total when 1 cc. of NaCl solution was used, by 1.2 per cent. Addition of water up to 6 cc. to the solution in the tube had no effect.

*Potassium Chloride.*—A similar series of twelve tests on a 0.1 N KCl solution (from Baker and Adamson, C.P. analyzed, recrystallized twice, dried 2 days at 115°, gave an average of

98.52 per cent with a maximum deviation from the average of 1.3 per cent.

*Potassium Phosphate.*—A 0.1 N solution from Baker and Adamson, c.p. analyzed  $\text{KH}_2\text{PO}_4$ , recrystallized four times, and dried at  $115^\circ$  for 24 hours gave as an average of three tests 98.53 per cent, with a maximum deviation from the average of 0.4 per cent.

*Mixtures of Sodium, Potassium, Calcium, and Magnesium.*—A calcium chloride solution was made from Eimer and Amend T.P. precipitated  $\text{CaCO}_3$ , dried for 2 days at  $115^\circ$  and dissolved in HCl. A  $\text{MgCl}_2$  solution was made from MgO, c.p., dissolved in HCl. From these solutions and the sources noted above various proportions of the bases were mixed, and the solutions run through the whole method. The actual amount found is given in the sixth column of Table II, while the "per cent of total found" was figured according to the method; *i.e.*, the established correction of 1.2 per cent was added to the total. In Table II the "time precipitated" column represents the time the solution was allowed to remain in the centrifuge tube before a sample was removed for evaporation on the steam bath; "cc. of solution" means the cc. removed for evaporation.

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# THE ACTION ON TYROSINE AND ON PHENYLAMINO-ACETIC ACID OF ACETIC ANHYDRIDE AND ACETONE IN THE PRESENCE OF PYRIDINE.

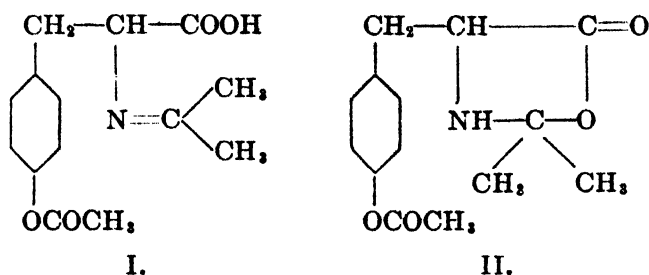
BY P. A. LEVENE AND ROBERT E. STEIGER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, July 6, 1927.)

There are recorded in literature several instances in which the acylation of amino acids in the presence of pyridine proceeded abnormally.<sup>1</sup> We have encountered an unexpected reaction in attempting to acetylate *l*-tyrosine suspended in a solution of pyridine in commercial acetic anhydride (containing 90 to 95 per cent of the pure anhydride). From the reaction product a crystalline substance,  $C_{14}H_{17}O_4N$ , melting at 122–123°C., was isolated. This compound contains at least one acetyl group (attached to the tyrosine hydroxyl) which is easily removed on boiling for a short time with 1 equivalent of 0.66 N sodium hydroxide. A compound,  $C_{12}H_{15}O_3N$ , is thus obtained.

On the basis of these preliminary results, the two possible structures I and II were considered for the compound  $C_{14}H_{17}O_4N$ .



These formulæ suggested the possibility that the compound is formed by condensation of acetyltyrosine with acetone.<sup>2</sup>

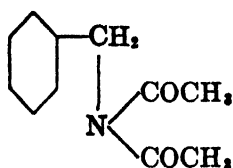
<sup>1</sup> Heller, G., and Tischner, W., *Ber. chem. Ges.*, 1910, xliii, 2574.

<sup>2</sup> The acetone may have been present already in the commercial impure anhydride which was used, or else formed in the course of the reaction.

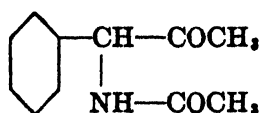
If the formation of the substance is actually such, then the reaction should be facilitated by the addition of acetone to the mixture used in the earlier experiments. The expectation was realized experimentally.

The same reaction was then carried out on phenylaminoacetic acid. In this case a crystalline compound,  $C_{11}H_{13}O_2N$ , melting between  $100-101^\circ C.$ , was obtained. Apparently this compound is of the same structural type as the one obtained from tyrosine. In their compositions, the two reaction products differ by the same value,  $C_3H_4$ , from those of the amino acids from which they are derived. (In the case of tyrosine it is of course necessary to allow for the acetyl group present in the hydroxyl.)

An abundant evolution of carbon dioxide (not measured, however) was observed during the reaction with phenylaminoacetic acid at  $80-90^\circ C.$  Therefore, for the derivative of this acid (and possibly for the tyrosine derivative, although no observations in this direction were made on tyrosine) one may also consider the possibility of the two following structures.



III.



IV.

Lack of time and of material prevent us from continuing this investigation at the present time. Hence an exhaustive study of the structures of the substances described and the part played by the additional acetone is postponed to a future date.

#### EXPERIMENTAL.

##### *I. Action of a Mixture of Commercial Acetic Anhydride and Pyridine on l-Tyrosine. (Preliminary Experiments.)*

1. 14 gm. of *l*-tyrosine,<sup>3</sup> 60 gm. of dry pyridine, and 100 gm. of commercial acetic anhydride (90 to 95 per cent) were heated for an hour in a bath maintained at  $80-90^\circ C.$ , the mixture being shaken from time to time. The tyrosine dissolved gradually.

<sup>3</sup> Obtained from silk.

The solution was then concentrated under reduced pressure to a thick syrup to which 150 cc. of water were added. After it was stirred, then left standing for a few hours, crystallization took place. The crude product was filtered off by suction, washed with water, and dried. Yield, 11.5 gm. Recrystallization from 24 cc. of acetone gave 6 gm. of colorless material which was further purified by recrystallization from 15 cc. of acetone. Melting point, 122–123°C.

No. 60.

$C_{14}H_{17}O_4N$  (263.22). Calculated. C 63.85, H 6.51, N 5.32.

Found. " 63.92, " 6.66, " 5.32 (Kjeldahl).  
" 5.31 (Dumas).

No. 60 recrystallized from boiling water gave No. 62, melting at 122–123°C.

No. 62.

C 63.63, H 6.56, N 5.39 (Kjeldahl).

2. Identical proportions were used as in the first experiment; the time of heating was reduced to 20 minutes and the temperature was increased to 100°C. The oil obtained by concentrating the mixture was shaken in a shaking machine with 150 cc. of water. Crystallization took place after standing overnight. 6.5 gm. of crude product thus obtained, when recrystallized from 70 cc. of boiling water in the presence of some norit, gave yellowish crystals melting at 122–123°C. The mother liquors of the first crop, when concentrated under reduced pressure, gave 4.6 gm. of crude product, thus making a total of 11.1 gm. It was fairly pure as it melted at 122–123°C., although sintering already had taken place at 115°C.

## *II. Action of Alkali on the Compound $C_{14}H_{17}O_4N$ .*

2.63 gm. (0.01 mol) of finely powdered product melting at 122–123°C. were boiled with 15 cc. of 0.66 N sodium hydroxide (1 equivalent) until dissolved. The solution was allowed to cool somewhat and while still warm 2 cc. of 5.0 N hydrochloric acid (1 equivalent) were added, and the mixture left to crystallize. The resulting 1.8 gm. of product were recrystallized from 15 cc.

of boiling water in the presence of some norit. Melting point, 163–165°C.

No. 63.

C 64.97, H 6.85, N 6.16 (Kjeldahl).

No. 63 was then recrystallized from boiling absolute alcohol. Melting point, 163–166°C.

No. 64.

$C_{12}H_{18}O_3N$ . (221.19).

Calculated. C 65.13, H 6.84, N 6.33.

Found. " 65.23, " 6.77, " 6.45 (Kjeldahl).

" 0.49 (Van Slyke amino).

### *III. Action of a Mixture of Commercial Acetic Anhydride, Acetone, and Pyridine on l-Tyrosine.*

37 gm. of *l*-tyrosine<sup>3</sup> (0.2 mol), 13 gm. of acetone (0.2 mol + 1 gm. excess), 79 gm. of dry pyridine, and 200 gm. of commercial acetic anhydride (90 to 95 per cent), placed in a 1 liter round bottom Pyrex flask, were heated for an hour in a bath kept at 80–90°C. The tyrosine dissolved gradually. Occasional shaking was found necessary. The reaction mixture was then concentrated to very small volume under reduced pressure. In order to remove residual acetic anhydride, a slow, continuous current of commercial xylene was introduced by means of a capillary extending below the surface of the oily residue. The temperature of the bath was kept around 95°C.; 150 cc. of xylene were thus vaporized during this distillation. The flask, the contents of which became partly solid on cooling, was kept under reduced pressure in a desiccator over paraffin until the crystals were free from xylene. They were then dissolved in 40 gm. of pure, boiling methylethyl ketone. The solution was filtered while hot and crystallization took place on seeding. The crystals which deposited after cooling overnight at 0°C. were filtered off by suction, were washed on the filter, first with a very small amount of pure ketone, then thoroughly with anhydrous ether, and were dried in a vacuum oven at 70°C. Yield, 16.8 gm. Recrystallization from 20 gm. of methylethyl ketone gave 14.2 gm. of crystals sintering very slightly above 115°C., and melting at 122–123°C. They were

finally purified by recrystallization from 32 gm. of boiling acetone. Melting took place sharply between 122–123°C.

Nos. 80, 74.

$C_{14}H_{17}O_4N$  (263.22).

Calculated. C 63.85, H 6.51, N 5.32.

Found. No. 80. " 63.93, " 6.64, " 5.29 (Kjeldahl).

" " 74. " 0 (Van Slyke amino).

#### IV. Action of a Mixture of Commercial Acetic Anhydride, Acetone, and Pyridine on Phenylaminoacetic Acid.

15 gm. of phenylaminoacetic acid<sup>4</sup> (0.1 mol), 7 gm. of acetone (0.1 mol + 1 gm. excess), 48 gm. of dry pyridine, and 100 gm. of acetic anhydride, placed in a 500 cc. round bottom Pyrex flask, were heated for an hour in a bath kept at 80°C. and shaken occasionally. Carbon dioxide escaped at the top of the reflux condenser. The liquor was concentrated under reduced pressure to a very viscous syrup. This residue yielded a crystalline mass, either spontaneously or when stirred with a small amount of acetone. After drying, it weighed 19.7 gm. It was crystallized from 10 gm. of boiling acetone. (Filtration of the hot solution must be carried out rapidly by suction as crystallization begins immediately.) The product was washed on the filter, first with a small amount of acetone, then with anhydrous ether to effect complete removal of the yellow coloring matter. Recrystallization from an equal weight of boiling absolute alcohol gave a product which, after drying at 70°C. (vacuum oven), melted between 100–101°C.

Nos. 79, 86.

$C_{11}H_{13}O_2N$  (191.17).

Calculated. C 69.08, H 6.85, N 7.33.

Found No. 79. " 69.44, " 7.02, " 7.33 (Kjeldahl).

" " 86. " 69.63,<sup>5</sup> " 7.24,<sup>5</sup> " 7.20 "

" 0.08 (Van Slyke amino).

<sup>4</sup> Prepared according to the directions given by Marvel, C. S., and Noyes, A. W., *J. Am. Chem. Soc.*, 1920, xlii, 2264; Ingersoll, A. W., and Adams, R., *J. Am. Chem. Soc.*, 1922, xlv, 2933, and purified by crystallization from boiling water.

<sup>5</sup> For combustion the substance was mixed in the boat with copper oxide.





# PENTAMETHYL *d*-MANNOSE AND PENTAMETHYL *d*-GALACTOSE AND THEIR DIMETHYL ACETALS.

BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, July 6, 1927.)

In a previous communication,<sup>1</sup> we reported on the preparation of pentamethylglucose and of its dimethyl acetal. The same method which led to the preparation of the above substances made possible the preparation of the corresponding derivatives of mannose and of galactose. The peculiarities of the two new aldehydic sugars were analogous to those of glucose. They concerned carbon atom (1). As in the case of the latter substance it was found: (1) That the pentamethyl mannose and galactose readily condensed with alcohol in the absence of catalysts to form acetals. On the other hand, their acetals were very readily hydrolyzed. (2) They reduced ammoniacal silver solution and alkaline permanganate solution in the cold, the latter nearly instantaneously.

Furthermore, it seemed of interest to compare the changes in the direction of rotation on the transformation of the pentamethyl sugars into their dimethyl acetals on one hand, and of those on transformation of the pentamethyl sugar acids into their salts, on the other.

In the free sugar acids the following observations were made. *d*-Gluconic and *d*-galactonic acids show a lower dextrorotation than their salts; *d*-mannonic acid on the other hand has a higher dextrorotation than its salts. The same relationship holds for all epimeric  $\alpha$ -hydroxy acids; namely, those having the hydroxyl on the same side as the  $\alpha$ -carbon atom of *d*-gluconic acid show a lower dextrorotation of the free acids (undissociated) than of their salts (ions); on the other hand, those having the hydroxyl on the same side as *d*-mannonic acid show a higher dextrorotation of the acids

<sup>1</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1926, lxi, 175.

than of their salts. As a rule, the rotations of the methyl or ethyl esters differ from those of the free acids in the same sense as the salts differ from the acids.

Exceptions to the above rule were observed in methoxy acids. Thus tetramethyl-*d*-mannonic acid had a lower rotation than its salt and in this respect behaved as tetramethyl-*d*-gluconic acid.

The data given below show that the three pentamethyl hexoses show a lower dextrorotation than the corresponding dimethyl acetals. In other words, their behavior is analogous to that of methylated hexonic acids.

	Pentamethyl derivative. $[\alpha]_D^{20}$	Dimethyl acetal. $[\alpha]_D^{20}$
Glucose.....	-35.4°	+15.0°
Galactose.....	-4.8	0
Mannose.....	+9.0	+19.3

#### EXPERIMENTAL.

*Diethylmercaptomannose*.—Although this compound has been already described by Fischer,<sup>2</sup> the method for its preparation will be given in detail inasmuch as directions given by Fischer require modification to give satisfactory results.

200 gm. of mannose are dissolved in 200 cc. of hydrochloric acid c.p. (sp. gr. 1.71 to 1.83) in a 2.5 liter bottle, and 200 cc. of technical ethyl mercaptan are added. The mixture is shaken vigorously by hand, the pressure being released occasionally. In 5 minutes a slight rise of temperature will occur accompanied by a slight change of color. A little cold water and ice are added. The contents of the bottle will nearly immediately solidify to a crystalline mass. More cold water is added and the diethylmercaptomannose filtered off. The yield of the crude dried product is 200 gm. It is recrystallized from hot 95 per cent alcohol.

If the mixture is shaken longer than mentioned above, the color soon darkens to a deep purple and the yield is considerably lowered.

*Pentamethyl Diethylmercaptomannose*.—Diethylmercaptomannose is methylated with dimethyl sulfate and sodium hydroxide as described in detail in a previous paper. The syrup obtained by

<sup>2</sup> Fischer, E., *Ber. chem. Ges.*, 1894, xxvii, 673.

this methylation is remethylated with sodium and methyl iodide under slightly modified conditions.

50 gm. of the partially methylated product are dissolved in anhydrous ether and allowed to react with metallic sodium. The ether is removed under diminished pressure. 20 cc. of methyl iodide dissolved in an equal volume of anhydrous ether are added with occasional shaking. The reaction is less violent than when undiluted methyl iodide is used. After about one hour the sodium iodide is precipitated with a large excess of ether. The fully methylated sugar is isolated as usual. The yield is 45 gm. The syrup is distilled; that fraction, 25 gm., boiling at 155–160°,  $p = 0.2$  mm., analyzed as follows:

0.1002 gm. substance: 0.1830 gm.  $\text{CO}_2$  and 0.0794 gm.  $\text{H}_2\text{O}$ .

0.1284 " " (Zeisel) required 16.9 cc. 0.1 N  $\text{AgNO}_3$ .

0.1742 " " : 0.2344 gm.  $\text{BaSO}_4$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6\text{S}_2$ . Calculated. C 50.20, H 9.00, S 18.00,  $\text{OCH}_3$  43.5.

Found. " 49.80, " 8.86, " 18.48, " 40.8.

It had the following rotation in methyl alcohol.

$$[\alpha]_D^{20} = \frac{+ 3.15^\circ \times 100}{1 \times 8} = + 39.4^\circ.$$

*Pentamethyl Mannose*.—The methylated mercaptomannose was hydrolyzed with mercuric chloride in methyl alcohol solution. The final product still contained a trace of sulfur and was distilled with a small quantity of potassium permanganate.

It distilled at 98–100°,  $p = 0.1$  mm., and analyzed as follows:

0.0937 gm. substance: 0.1818 gm.  $\text{CO}_2$  and 0.0732 gm.  $\text{H}_2\text{O}$ .

0.1036 " " (Zeisel) required 20.60 cc. 0.1 N  $\text{AgNO}_3$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6$ . Calculated. C 52.80, H 8.80,  $\text{OCH}_3$  62.00.

Found. " 52.91, " 8.74, " 61.64.

It had the following rotation in acetylene tetrachloride.

$$[\alpha]_D^{20} = \frac{+ 0.58^\circ \times 100}{1 \times 6.4} = + 9.1^\circ.$$

In cold methyl alcohol the initial rotation is  $[\alpha]_D^{15} = +8.00^\circ$ ; allowed to stand at room temperature, in 10 minutes it is  $[\alpha]_D^{20} = +12.30^\circ$  and comes to equilibrium at  $[\alpha]_D^{20} = +17.8^\circ$ . Pentamethyl-

## 698 Pentamethyl *d*-Mannose and *d*-Galactose

glucose reduces Fehling's solution on warming and alkaline silver nitrate in the cold.

*Pentamethyl Mannose Dimethyl Acetal*.—This was prepared similarly to the corresponding methylated glucose compound. It distilled at 112–114°C.,  $p = 0.1$  mm., and analyzed as follows:

5.855 mg. substance: 11.278 mg.  $\text{CO}_2$  and 5.001 mg.  $\text{H}_2\text{O}$ .

0.1120 gm. " (Zeisel) required 25.20 cc. 0.1  $\text{N}$   $\text{AgNO}_3$ .

$\text{C}_{13}\text{H}_{23}\text{O}_7$ . Calculated. C 52.70, H 9.50,  $\text{OCH}_3$  73.20.

Found. " 52.57, " 9.55, " 69.70.

It had the optical rotation in methyl alcohol

$$[\alpha]_D^{20} = \frac{+ 1.42^\circ \times 100}{1 \times 6.7} = + 21.2^\circ;$$

and in acetylene tetrachloride,

$$[\alpha]_D^{20} = \frac{+ 1.1^\circ \times 100}{1 \times 5.7} = + 19.3^\circ.$$

*Pentamethyl Galactose*.—This was prepared entirely on the lines of the preceding sugar. Details of the methods will therefore be omitted.

*Pentamethyl Diethylmercaptogalactose*.—This substance distills at 155–160°C.,  $p = 0.2$  mm. It analyzed as follows:

6.412 mg. substance: 11.77 mg.  $\text{CO}_2$  and 5.03 mg.  $\text{H}_2\text{O}$ .

0.1248 gm. " : 0.1690 gm.  $\text{BaSO}_4$ .

0.1403 " " (Zeisel) required 19.0 cc. 0.1  $\text{N}$   $\text{AgNO}_3$ .

$\text{C}_{15}\text{H}_{22}\text{O}_5\text{S}_2$ . Calculated. C 50.20, H 9.00, S 18.00,  $\text{OCH}_3$  43.50.

Found. " 50.06, " 8.78, " 18.60, " 41.91.

In a concentration of 11 per cent this compound does not show any optical rotation in methyl alcohol.

*Pentamethyl Galactose*.—This substance analyzed as follows:

0.0950 gm. substance: 0.1830 gm.  $\text{CO}_2$  and 0.0754 gm.  $\text{H}_2\text{O}$ .

0.1150 " " (Zeisel) required 22.65 cc. 0.1  $\text{N}$   $\text{AgNO}_3$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6$ . Calculated. C 52.80, H 8.80,  $\text{OCH}_3$  62.00.

Found. " 52.52, " 8.88, " 61.00.

In acetylene tetrachloride the rotation was

$$[\alpha]_D^{20} = \frac{- 0.20^\circ \times 100}{1 \times 4.12} = - 4.8^\circ.$$

In methyl alcohol ( $c = 4.54$ ,  $l = 1$ ) there was no appreciable rotation noted at the time of mixing. After 1 hour  $[\alpha]_D^{20} = -6^\circ$  which became constant at  $[\alpha]_D^{20} = -10^\circ$ .

In methyl alcohol containing 0.2 per cent hydrochloric acid this rotation became immediately  $[\alpha]_D^{20} = -10^\circ$ .

Pentamethyl galactose shows similar behavior to the corresponding mannose and glucose compounds.

*Pentamethyl Galactose Dimethyl Acetal*.—This substance distilled at  $118\text{--}120^\circ\text{C}$ .,  $p = 0.6$  mm. It analyzed as follows:

12.890 mg. substance: 24.949 mg.  $\text{CO}_2$  and 10.690 mg.  $\text{H}_2\text{O}$ .

0.0970 gm. " (Zeisel) required 22.3 cc. 0.1 N  $\text{AgNO}_3$ .

$\text{C}_{13}\text{H}_{28}\text{O}_7$ . Calculated. C 52.70, H 9.50,  $\text{OCH}_3$  73.20.

Found. " 52.77, " 9.28, " 71.20.

There was no noticeable rotation in acetylene tetrachloride ( $c = 7$ ,  $l = 1$ ).



## DIACETONE GLUCOSE.

### IV. $\alpha$ - AND $\beta$ -ISOMERS OF 3, 5, 6-TRIMETHYLMETHYLGLUCOSIDE AND OF 2, 3, 5, 6-TETRAMETHYLMETHYLGLUCOSIDE.

BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, July 6, 1927.)

In a previous paper<sup>1</sup> it was shown that two trimethylmethylglucosides could be prepared from 1,2-monoacetone-3,5,6-trimethylglucose. Of the two, the lower boiling is dextrorotatory and the higher boiling is levorotatory. At the time of that publication the highest value obtained for the dextrorotatory substance was  $[\alpha]_D^{20} = +75^\circ$  and for the levorotatory  $[\alpha]_D^{20} = -45^\circ$ , both in methyl alcoholic solution. The nature of the isomerism remained to be established.

Through fractional distillation of a larger quantity of material it was possible to isolate a dextrorotatory fraction with a specific rotation of  $[\alpha]_D^{20} = +93^\circ$  (boiling at 105–109°C. at 0.4 mm. pressure) and a levorotatory fraction with a specific rotation of  $[\alpha]_D^{20} = -87^\circ$  (boiling at 145–150°C.,  $p=0.2$  mm.). It is now found that the dextro- and levorotatory substances are the  $\alpha$ - and  $\beta$ -isomers of 3,5,6-trimethylmethylglucoside. This conclusion was reached on the basis of the following considerations.

1. It is known that in a solution of methyl alcohol containing 0.1 per cent of hydrogen chloride levo- and dextro-glucosides undergo interconversion (mutarotate). If the two 3,5,6-trimethylmethylglucosides are  $\alpha$ - and  $\beta$ -isomers, then the equilibrium forms obtained from each should possess the identical optical rotation.

2. The rates of the mutarotations of the two forms should be identical under identical conditions.

3. If the dextro- and levorotatory substances are the  $\alpha$  and  $\beta$  forms of the 3,5,6-trimethylmethylglucoside, they should each

<sup>1</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1926, lxx, 343.



yield 3,5,6-trimethylglucose having the identical equilibrium rotation.

4. The sugars obtained in (3) should on oxidation yield the identical monocarboxylic acid.

In a general way the four requirements necessary to establish the postulated relationship of the two forms were fulfilled. Some minor discrepancies may be explained by the fact that each isomer was obtained in the form of a syrup and although the quantities of materials at our disposal were considerable, yet they were not such as to permit the assurance that each form was uncontaminated with some of its isomer, or with a small quantity of some extraneous impurity.

In detail the results were the following:

1. The dextrorotatory form with a rotation of  $[\alpha]_D^{20} = +93^\circ$  on hydrolysis gave a product with a rotation of  $[\alpha]_D^{20} = -12.5^\circ$ . The levorotatory substance with a rotation of  $[\alpha]_D^{20} = -87^\circ$  gave on hydrolysis a material with a slight dextrorotation. On redistillation the first few drops were dextrorotatory and the bulk had a rotation of  $[\alpha]_D^{20} = -10.0^\circ$ . The dextrorotatory substance seems to have the composition of 3,5,6-trimethylmethylglucoside, but the minute yield of it did not permit further analysis.

2. On oxidation of the sugar obtained from the dextrorotatory glucoside an acid was obtained of which the sodium salt had the rotation of  $[\alpha]_D^{20} = +31^\circ$ . On identical treatment the sodium salt of the acid obtained from the levorotatory glucoside had a rotation of  $[\alpha]_D^{20} = +31.3^\circ$ . On hydrolysis and subsequent oxidation of monoacetone-3,5,6-trimethylglucose an acid was obtained of which the sodium salt had the rotation of  $[\alpha]_D^{20} = +29.7^\circ$ .

3. On treatment at room temperature with methyl alcohol containing 0.1 per cent of hydrogen chloride, a 10 per cent solution of the dextrorotatory glucoside gave a product with a specific rotation of  $[\alpha]_D^{20} = -13^\circ$  and a 20 per cent solution a product with  $[\alpha]_D^{20} = -14^\circ$ . The levorotatory fraction under the same conditions gave products with specific rotations of  $[\alpha]_D^{20} = -12.5^\circ$  and  $-5^\circ$  respectively.

4. The rates of mutarotation in methyl alcoholic solution in the presence of 0.1 per cent of hydrogen chloride and at the temperature of  $23^\circ\text{C}$ . calculated by the expression  $(k_1 + k_2) = \frac{1}{t} \log_{10} \frac{r_\infty - r_0}{r_\infty - r_t}$  were for the dextrorotatory form  $985 \times 10^{-5}$  and for the levorota-

tory  $804 \times 10^{-5}$  in 10 per cent concentration. The values were  $791 \times 10^{-5}$  and  $840 \times 10^{-5}$  respectively in a 20 per cent solution. The small difference in the values of the  $\alpha$  and  $\beta$  forms may be easily explained by the slight impurity in the levorotatory substance. The values are close enough to show that the two velocities are of the same order of magnitude. It may be added here that the velocities of interconversion of the  $\alpha$  and  $\beta$  forms of  $<1,5>$  tetramethylmethylglucosides are of an entirely different order of magnitude.<sup>2</sup>

Thus, the above data seem to justify the conclusion that the levo- and dextrorotatory fractions of the material which are obtained on the conversion of monoacetone-trimethylglucose into trimethylmethylglucosides represent the  $\alpha$  and  $\beta$  forms of 3,5,6-trimethylmethylglucoside.

We hoped, however, that pure fractions could be obtained after the conversion of the trimethylglucosides into the tetramethyl derivatives. This expectation was based on the fact that the higher boiling fractions of the trimethylglucosides always contained a small proportion of decomposition product. Inasmuch as the tetramethylglucosides boil at lower temperatures than trimethyl derivatives it was natural to hope for less decomposition on the distillation of the former. In reality the data obtained on the two forms of the tetramethylmethylglucoside were practically of the same character as those obtained from the observations on the trimethyl derivatives.

The details of the observations were the following:

1. The dextrorotatory material had an optical rotation of  $[\alpha]_D^{20} = +104.36^\circ$ . The fraction with the highest negative rotation had  $[\alpha]_D^{20} = -64^\circ$ .

2. On hydrolysis the dextrorotatory substance gave a tetramethylglucose with a rotation of  $[\alpha]_D^{20} = -17.1^\circ$  (in benzene).

The levorotatory fraction as in the case of the 3,5,6-trimethylglucose contained a dextrorotatory impurity. Thus the product of hydrolysis had a rotation of  $+5^\circ$ , which on distillation yielded a minute dextrorotatory fraction and the bulk with  $[\alpha]_D^{20} = -13.5^\circ$ .

3. In methyl alcoholic solution at  $24^\circ\text{C}$ . in the presence of 0.1 per cent of hydrogen chloride the dextrorotatory form mutarotated

<sup>2</sup> Irvine, J. C., and Cameron, A., *J. Chem. Soc.*, 1904, lxxxv, 900.

giving an equilibrium product with a rotation of  $[\alpha]_D^{24} = -24.15^\circ$  and the levorotatory substance under identical conditions yielded a product with a specific rotation of  $-17.75^\circ$ .

4. The rates of mutarotation calculated as in the case of the trimethylmethylglucosides were  $(k_1 + k_2)$  for the dextro form  $171 \times 10^{-5}$  and  $169 \times 10^{-5}$  for the levo form.

Thus the fractions obtained on the fractionation of the tetramethylmethylglucosides were of the same degree of purity as those obtained on distillation of the 3,5,6-trimethylmethylglucosides and the observations on the tetramethylmethylglucosides confirm the conclusion that the dextro- and levorotatory substances are the  $\alpha$  and  $\beta$  forms of the methylated  $< 1,4 >$  methyl glucoside.

The existence of two pairs of  $\alpha$  and  $\beta$  forms of glucosides each differing from the other only in ring structure permits the analysis of the influence of the ring structure on the magnitude of the rotation of carbon atom (1).

For the glucoses, to the common forms the  $< 1,5 >$  ring structure and to the  $\gamma$  forms the  $< 1,4 >$  ring structure may be assigned. Calculating according to Hudson's rule one obtains twice the value of carbon atom (1) of the common forms of tetramethylmethylglucoside to be 34,230 and of the  $\gamma$  forms 42,000. It may be noted that the values of rotations of carbon atom (1) for the trimethyl and tetramethyl derivatives are practically identical inasmuch as for the 2,3,4-trimethylmethylglucoside twice the value of carbon atom (1) is 34,692 and for 3,5,6-trimethylmethylglucoside the corresponding value is 42,480.

These observations may be significant in connection with another analogous pair each consisting of an  $\alpha$  and  $\beta$  form of a sugar derivative, namely with the acetyl galactoses. Two pairs of  $\alpha$ - and  $\beta$ -pentacetates were prepared by Hudson and Parker<sup>3</sup> and Hudson and Johnson.<sup>4</sup> Twice the value of the rotation of carbon atom (1) of the first pair is 32,700 and of the second is 40,200. Levene and Sobotka<sup>5</sup> were inclined to assign to the first pair the  $< 1,5 >$  structure and to the second pair the  $< 1,4 >$  ring structure. This conclusion gains in probability on the basis of the above observations on the two pairs of tetramethylmethylglucosides. It is,

<sup>3</sup> Hudson, C. S., and Parker, H. O., *J. Chem. Soc.*, 1915, xxxvii, 1589.

<sup>4</sup> Hudson, C. S., and Johnson, J. M., *J. Chem. Soc.*, 1916, xxxviii, 1223.

<sup>5</sup> Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxxvii, 759.

however, realized that all such conclusions can be regarded only as tentative so long as the number of observations is small.

#### CONCLUSION.

The  $\alpha$ - and  $\beta$ -isomers of 3,5,6-trimethylmethylglucosides (the  $\gamma$  forms) and of  $\alpha$ - and  $\beta$ -2,3,5,6-tetramethylmethylglucosides were obtained.

The <1,4> methylated glucosides in addition to the peculiarities previously pointed out differ from the common forms in the following respects. (1) The interconversion of the  $\alpha$ - and  $\beta$ -isomers proceeds at a higher rate in the  $\gamma$  forms. (2) The rate of hydrolysis of the  $\gamma$ -glucosides is higher than that of the corresponding common forms. (3) The rotation of the carbon atom (1) of the  $\gamma$  forms has a higher value than that of the common forms.

#### EXPERIMENTAL.

*3,5,6-Trimethylmonoacetone Glucose*.—Monoacetone glucose was methylated with dimethyl sulfate and sodium hydroxide as described in a previous publication. In order to insure complete methylation, the crude syrup was remethylated in portions of 50 gm. with 20 gm. of silver oxide and 20 gm. of methyl iodide.

The product then distilled at 110–115°C.,  $p=0.2$  mm., and had a rotation of

$$[\alpha]_D^{20} = \frac{-1.26^\circ \times 100}{1 \times 4.64} = -27.2^\circ.$$

It analyzed as follows:

0.0971 gm. substance: 0.1950 gm. CO<sub>2</sub> and 0.0754 gm. H<sub>2</sub>O.

0.1542 " " (Zeisel) required 17.60 cc. 0.1 N AgNO<sub>3</sub>.

C<sub>12</sub>H<sub>22</sub>O<sub>6</sub>. Calculated. C 54.96, H 8.46, OCH<sub>3</sub> 35.50.

Found. " 54.76, " 8.68, " 35.38.

*3,5,6-Trimethylmethylglucosides*.—50 gm. of trimethylmonoacetone glucose dissolved in 500 cc. of methyl alcohol containing 0.5 per cent of hydrogen chloride were heated in an autoclave at 100°C. for 24 hours and further treated as previously described. The syrup was subjected to fractional distillation.

Boiling point. °C.	Pressure. mm.	$[\alpha]_D^{20}$ degrees
110-114	0.4	+85
114-120	0.4	+69.9
120-130	0.4	+54.8
130-140	0.4	-4
140-150	0.4	-38
150-155	0.4	-85

Several preparations having the higher dextro- and levorotations were combined and refractionated. In this manner two fractions were finally obtained: (1) boiling at 105-109°C.,  $p=0.4$  mm.,

TABLE I.

*Rate of Mutarotation of 3,5,6-Trimethylmethylglucosides in Methyl Alcohol Containing 0.2 Per Cent HCl.*

$t = 24.2^\circ\text{C}$ .  $c = 10$  per cent.

$\alpha$ form.			$\beta$ form.		
Time.	$[\alpha]_D^{24.2}$	$(k_1 + k_2) \times 10^5$	Time.	$[\alpha]_D^{24.2}$	$(k_1 + k_2) \times 10^5$
<i>min.</i>			<i>min.</i>		
0	+83.5		0	-80.0	
10	+63.6	1000	11	-67.0	845
20	+48.0	995	20	-59.3	800
30	+35.5	995	25	-55.4	787
41	+24.0	1010	35	-48.8	800
58	+12.2	1000	99	-23.0	830
91	-1.8	910	133	-19.0	764
$\infty$	-13.0		$\infty$	-12.5	
Average.....					804

which had  $[\alpha]_D^{20} = \frac{+2.86^\circ \times 100}{1 \times 3.08} = +93^\circ$  in methyl alcohol; (2)

boiling at 145-150°C.,  $p=0.2$  mm.,  $[\alpha]_D^{20} = \frac{-2.44^\circ \times 100}{1 \times 2.804} = -87^\circ$  in methyl alcohol.

Fractions 1 and 2 analyzed as follows:

1. 0.0933 gm. substance: 0.1744 gm.  $\text{CO}_2$  and 0.0712 gm.  $\text{H}_2\text{O}$ .
2. 0.0954 " " : 0.1764 " " " 0.0726 " "
1. 0.1054 " " (Zeisel) required 17.9 cc. 0.1 N  $\text{AgNO}_3$ .
2. 0.1186 " " " " 19.8 " 0.1 " "

$C_{16}H_{20}O_6$ .	Calculated.	C 50.95, H 8.47, $OCH_3$ 52.50.
1. Found.	"	50.96, " 8.53, " 52.60.
2. " "	"	50.42, " 8.51, " 51.75.

*Interconversion of the  $\alpha$ - and  $\beta$ -Glucosides.*—The rotation of the two glucosides dissolved in methyl alcohol containing 0.2 per cent hydrochloric acid changes rapidly at room temperature, as shown in Tables I and II.

This property may be utilized to obtain either the  $\alpha$  or  $\beta$  form as desired. The levo-glucoside being less accessible, the dextro frac-

TABLE II.

*Rate of Mutarotation of 3,5,6-Trimethylmethylglucosides in Methyl Alcohol Containing 0.2 Per Cent HCl.*

$t = 24^\circ C.$   $c = 20$  per cent.

$\alpha$ form.			$\beta$ form.		
Time.	$[\alpha]_D^{20}$	$(k_1 + k_2) \times 10^6$	Time.	$[\alpha]_D^{20}$	$(k_1 + k_2) \times 10^6$
<i>min.</i>			<i>min.</i>		
0	+85.92		0	-67.60	
24	+50.92	780	19	-47.25	900
29	+45.20	784	42	-32.30	860
35	+38.75	793	70	-21.00	840
45	+30.00	769	92	-15.65	830
67	+15.00	802	116	-12.55	790
82	+7.60	800	$\infty$	-5.00	
92	+3.80	814			
$\infty$	-14.00				
Average.....		791			840

tion was converted into the equilibrium mixture and the levo-glucoside isolated by fractional distillation.

10 gm. of 3,5,6-trimethylmethylglucoside with  $[\alpha]_D^{20} = +89^\circ$  were dissolved in 100 cc. of methyl alcohol containing 0.2 per cent hydrogen chloride, and allowed to stand overnight. The solution was neutralized with sodium methylate, concentrated under diminished pressure, taken up in ether, filtered, and after removal of the solvent, fractionated.

- 4 gm. distilled at  $105-125^\circ C.$ ,  $p = 0.04$  mm.,  $[\alpha]_D^{20} = +50^\circ$ .
- 5 " " "  $155-165^\circ$  " "  $= 0.05$  "  $[\alpha]_D^{20} = -87^\circ$ .

Fraction 1 was again treated with methyl alcohol containing hydrogen chloride and in this manner further quantities of the levo-glucoside were isolated.

*Hydrolysis of the Glucosides.*—Heating the glucosides in boiling water with 8 per cent hydrochloric acid causes destruction of the sugar. The glucosides are readily hydrolyzed by heating in boiling water with 0.5 per cent hydrochloric acid. 7 gm. of glucoside with a rotation of  $[\alpha]_D^{20} = +93^\circ$  were heated with 70 cc. of 0.5 per cent hydrochloric acid for 1 hour. The rotation of the solution

TABLE III.

*Rate of Mutarotation of 2,3,5,6-Tetramethylmethylglucosides in Methyl Alcohol Containing 0.2 Per Cent HCl.*

$t = 24.8^\circ\text{C. } c = 20 \text{ per cent.}$

$\alpha$ form.			$\beta$ form.		
Time.	$[\alpha]_D^{24.8}$	$(k_1 + k_2) \times 10^5$	Time.	$[\alpha]_D^{24.8}$	$(k_1 + k_2) \times 10^5$
<i>min.</i>			<i>min.</i>		
0	+104.3		0	-64.0	
8	+100.4	161	10	-62.5	143
23	+93.3	170	15	-61.7	144
48	+81.6	180	25	-59.4	179
63	+76.2	175	40	-57.2	171
83	+69.7	167	70	-53.6	169
$\infty$	-22.5		120	-49.0	141
			$\infty$	-17.7	
Average.....					158
		170			

was then  $[\alpha]_D^{20} = -0.5^\circ$ . The acid was neutralized with barium carbonate. The filtrate was concentrated under diminished pressure and the sugar extracted with ether. The weight of the syrup was 5 gm. Prior to distillation but heated at  $100^\circ\text{C.}$  at 0.02 mm. pressure, the syrup had a specific rotation of  $[\alpha]_D^{20} = -7.5^\circ$ . It distilled at  $155^\circ\text{C.}$ ,  $p=0.02 \text{ mm.}$ , and had the following rotation in methyl alcohol.

$$[\alpha]_D^{20} = \frac{-1.0^\circ \times 100}{1 \times 8.1} = -12.3^\circ.$$

It analyzed as follows:

4.174 mg. substance: 7.401 mg. CO<sub>2</sub> and 3.242 mg. H<sub>2</sub>O.  
 0.1084 gm. " (Zeisel) required 14.50 cc. 0.1 N AgNO<sub>3</sub>.  
           C<sub>9</sub>H<sub>11</sub>O<sub>6</sub>. Calculated. C 48.65, H 8.11, OCH<sub>3</sub> 41.9.  
                                 Found. " 48.35, " 8.69, " 41.4.

6 gm. of the glucoside with  $[\alpha]_D^{20} = -80^\circ$  were hydrolyzed in 60 cc. 0.5 per cent hydrochloric acid for 1 hour. In this instance the rotation was  $[\alpha]_D^{20} = +0.5^\circ$ .

The syrup which was isolated as usual was then distilled. The first few drops which came over at 150–155°C., p. = 0.8 mm., had a rotation of  $[\alpha]_D^{20} = +18.9^\circ$ . The largest part distilled at 155°C., p = 0.2 mm., and had a rotation of  $[\alpha]_D^{20} = -10^\circ$ .

This fraction analyzed as follows:

7.957 mg. substance: 14.145 mg. CO<sub>2</sub> and 5.800 mg. H<sub>2</sub>O.  
 0.1110 gm. " (Zeisel) required 14.70 cc. 0.1 N AgNO<sub>3</sub>.  
           C<sub>9</sub>H<sub>11</sub>O<sub>6</sub>. Calculated. C 48.65, H 8.11, OCH<sub>3</sub> 41.90.  
                                 Found. " 48.47, " 8.15, " 41.05.

Neither sugar reduced Fehling's solution immediately in the cold.

*Oxidation of Sugar.*—Isolation of the lactones. The syrups obtained by hydrolysis of the dextro- and levo-glucosides were oxidized with bromine at 35–40°C. and the lactone isolated as usual. The methoxyl determinations gave the following results.

1. From the glucoside with  $[\alpha]_D^{20} = +93^\circ$ .
2. " " " "  $[\alpha]_D^{20} = -87^\circ$ .
1. 0.1034 gm. substance (Zeisel) required 14.5 cc. 0.1 N AgNO<sub>3</sub>.
2. 0.1312 " " " " 18.0 " 0.1 " "
- C<sub>9</sub>H<sub>11</sub>O<sub>6</sub>. Calculated. OCH<sub>3</sub> 42.30.
1. Found. " 43.40.
2. " " 42.76.

The rotation of the sodium salt of the acid obtained by dissolving the weighed lactone in the calculated amount of sodium hydroxide was as follows:

$$1. \quad [\alpha]_D^{20} = \frac{+1.55^\circ \times 100}{1 \times 5.00} = +31.0^\circ.$$

$$2. \quad [\alpha]_D^{20} = \frac{+1.25^\circ \times 100}{1 \times 4.00} = +31.3^\circ.$$



The rotation of the sodium salt of the acid obtained by hydrolysis and oxidation of 3,5,6-trimethylmonoacetone glucose reported in a previous paper was

$$[\alpha]_D^{20} = \frac{+1.45^\circ \times 100}{1 \times 4.88} = +29.7^\circ.$$

*Conversion of  $\alpha$ - and  $\beta$ -3,5,6-Trimethylmethylglucosides into the 2,3,5,6-Tetramethylmethylglucosides.*—The further methylation of the trimethylmethylglucosides by the Freudenberg method with sodium and methyl iodide is accompanied by considerable destruction of the sugar. Likewise methylation with silver oxide and methyl iodide in the usual proportions gives very poor yields. Methylation proceeds smoothly if a large excess of methyl iodide is used as solvent.

10 gm. of the dextro- or levo-3,5,6-trimethylglucosides were methylated with 120 gm. of methyl iodide and 50 gm. of silver oxide. The latter was added in small portions.

From the trimethylmethylglucoside with  $[\alpha]_D^{20} = +93^\circ$ , a tetramethylmethylglucoside was obtained having  $[\alpha]_D^{20} = \frac{+4.12^\circ \times 100}{1 \times 3.96} = +104^\circ$  in methyl alcohol. This distilled at  $105^\circ\text{C}$ .,  $p=0.2$  mm., and analyzed as follows:

0.0970 gm. substance: 0.1864 gm.  $\text{CO}_2$  and 0.759 gm.  $\text{H}_2\text{O}$ .

0.1260 " " (Zeisel) required 25.2 cc. 0.1 N  $\text{AgNO}_3$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6$ . Calculated. C 52.80, H 8.80,  $\text{OCH}_3$  62.00.

Found. " 52.60, " 9.06, " 61.62.

From the trimethylmethylglucoside with  $[\alpha]_D^{20} = -80^\circ$  a tetramethylmethylglucoside was obtained which had a rotation in methyl alcohol of

$$[\alpha]_D^{20} = \frac{-2.60^\circ \times 100}{1 \times 4.06} = -64^\circ.$$

It distilled at  $105^\circ\text{C}$ .,  $p=0.2$  mm., and analyzed as follows:

0.1020 gm. substance: 0.1948 gm.  $\text{CO}_2$  and 0.0808 gm.  $\text{H}_2\text{O}$ .

0.1134 " " (Zeisel) required 22.7 cc. 0.1 N  $\text{AgNO}_3$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6$ . Calculated. C 52.80, H 8.80,  $\text{OCH}_3$  62.00.

Found. " 52.40, " 8.76, " 62.05.

*Interconversion of the  $\alpha$ - and  $\beta$ -Tetramethylmethylglucosides.*—The rotations of the two tetramethylmethylglucosides in methyl

alcohol containing 0.2 per cent hydrogen chloride change quite rapidly at room temperature. The equilibrium rotation for the dextro form is  $[\alpha]_D^{24.8} = -22.5^\circ$  and for the levo form  $[\alpha]_D^{24.8} = -17.7^\circ$ . The rate of change is shown in Table III.

*Hydrolysis of the  $\alpha$ - and  $\beta$ -Tetramethylmethylglucosides.*—8 gm. of tetramethylmethylglucoside with  $[\alpha]_D^{20} = +101^\circ$  were hydrolyzed with 150 cc. of 0.1 N hydrochloric acid in boiling water. After 45 minutes the rotation became constant at  $[\alpha]_D^{20} = -10^\circ$ . The sugar was isolated as usual and the syrup amounting to 7 gm. distilled at  $122^\circ\text{C}$ .,  $p=0.4$  mm.

It analyzed as follows:

0.1009 gm. substance: 0.1884 gm.  $\text{CO}_2$  and 0.0764 gm.  $\text{H}_2\text{O}$ .  
 0.1138 " " (Zeisel) required 19.5 cc. 0.1 N  $\text{AgNO}_3$ .  
 $\text{C}_{10}\text{H}_{20}\text{O}_6$ . Calculated. C 50.95, H 8.47,  $\text{OCH}_3$  52.50.  
 Found. " 50.91, " 8.47, " 53.10.

The rotation in methyl alcohol was

$$[\alpha]_D^{20} = \frac{-1.25^\circ \times 100}{1 \times 6.1} = -20.5^\circ.$$

In benzene the rotation was

$$[\alpha]_D^{20} = \frac{-0.80^\circ \times 100}{1 \times 4.68} = -17.1^\circ.$$

This value agrees with that found by Irvine.<sup>6</sup>

The tetramethylmethylglucoside with  $[\alpha]_D^{20} = -64^\circ$  (7 gm.) was hydrolyzed under the same conditions. The syrup which was recovered (6 gm.) had a rotation of  $[\alpha]_D^{20} = -4.8^\circ$ . This was distilled. A small quantity distilled at  $125^\circ\text{C}$ .,  $p=0.8$  mm., and had  $[\alpha]_D^{20} = +7^\circ$ . The remainder distilled at  $122^\circ\text{C}$ .,  $p=0.05$  mm., and had  $[\alpha]_D^{20} = -13.5^\circ$ .

This analyzed as follows:

0.1045 gm. substance: 1.1944 gm.  $\text{CO}_2$  and 0.0780 gm.  $\text{H}_2\text{O}$ .  
 0.1178 " " (Zeisel) required 19.80 cc. 0.1 N  $\text{AgNO}_3$ .  
 $\text{C}_{10}\text{H}_{20}\text{O}_6$ . Calculated. C 50.95, H 8.47,  $\text{OCH}_3$  52.50.  
 Found. " 50.73, " 8.36, " 52.10

Neither the sugar obtained from the dextro- or levo-glucoside reduced Fehling's solution at ordinary temperature but both did so very strongly on warming.

<sup>6</sup> Irvine, J. C., Fyffe, A. W., and Hogg, T. P., *J. Chem. Soc.*, 1915, cvii, 524.



## NOTE ON THE PREPARATION OF CEPHALIN.

BY P. A. LEVENE AND IDA P. ROLF.

*(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)*

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The present note is prompted by the same motive as the recent note on the preparation of lecithin; namely, by the desire to offer a simple process of preparing cephalin to those who need the material for biological experiments. Judging by the request for samples of lecithin and cephalin, the number of investigators in need of the materials is considerable.

None of the methods of preparing the so called "cephalin" (a mixture of unaltered cephalin with lysocephalin) was satisfactory, inasmuch as success in the preparation of samples free from non-amino nitrogen was a matter of chance. Often products were obtained containing 80 or 90 per cent of their nitrogen as amino nitrogen, but these could not be improved further by any of the older procedures.

Cephalin preparations free from non-amino nitrogen can be rapidly prepared in the following way.

40.0 pounds of brain tissue, freed from membranes, are minced in a hashing machine and then dried in a vacuum drier. The dry material is then pulverized and the drying is continued. The product is exhaustively extracted with acetone. This extraction requires about 20 liters of acetone. The residue is freed from acetone in the vacuum drier and thoroughly extracted with 95 per cent alcohol. (30 liters of alcohol are required.) The residue after this extraction is extracted with 20 liters of ether, and the ethereal extract concentrated to small volume and allowed to stand at 0°C. overnight to allow the contaminating "white matter" to settle out. The latter is removed by centrifugalization and the supernatant liquid poured into 98.5 per cent alcohol warmed to 60°C. The precipitate thus formed is dissolved in ether and the

ethereal solution is again allowed to stand at 0°C. overnight. The operations of precipitation with alcohol and redissolving in ether are continued as long as the ethereal extract on standing deposits a sediment of white matter.

The product as finally formed by precipitation from alcohol contains no non-amino nitrogen. The yield is 18.0 gm.

The following is the analysis of one of several samples prepared in this manner.

0.0973 gm. substance: 0.2144 gm. CO<sub>2</sub> and 0.0844 gm. H<sub>2</sub>O. C 60.9, H 9.70.

0.1932 gm. substance required for neutralization 2.49 cc. of 0.1 N acid.  
N 1.80.

0.2898 gm. substance: 0.0372 gm. Mg<sub>3</sub>P<sub>2</sub>O<sub>7</sub>. P 3.58.

The substances contained practically no non-amino nitrogen.

## STUDIES ON RACEMIZATION.

### V. THE ACTION OF ALKALI ON GELATIN.

BY P. A. LEVENE AND LAWRENCE W. BASS.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

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It has been shown by Levene and Pfaltz<sup>1</sup> that ketopiperazines undergo racemization on standing with dilute alkali (0.1 N) when the concentration of alkali is low (1 equivalent). On the other hand, when stronger alkali (1.0 N, 5 to 15 equivalents) is used, hydrolysis of the ketopiperazines into the corresponding dipeptides proceeds so rapidly that the component amino acids escape racemization. Inasmuch as the possibility of the presence of ketopiperazines in the protein molecule has often been suggested, it seemed desirable to test the action of alkali on proteins. Gelatin was the first to be studied in connection with the present inquiry, since several ketopiperazines have been isolated from the products of enzymatic hydrolysis of this protein.

The interpretation of any change in the optical rotation of a protein is not a simple matter, inasmuch as the protein molecule readily undergoes hydrolysis when subjected to the action of alkali. This hydrolysis may bring about a change in rotation in one direction or the other, depending on the direction of the rotation of the hydrolytic products. In other words, the action of alkali on a protein is too complex for analysis. It is much simpler to bring about a complete hydrolysis of the reaction product of alkali on the protein and to observe the rotation of the mixture of amino acids thus obtained.

Inasmuch as some amino acids contained in gelatin are dextro-rotatory while others are levorotatory the following three results

<sup>1</sup> Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661; *J. Gen. Physiol.*, 1925, viii, 183; *J. Biol. Chem.*, 1926, lxviii, 277; 1926, lxx, 219.

are possible. (1) All the amino acids undergo equal racemization; the numerical value of the rotation of the mixture obtained from the racemized protein should be lower than that obtained by simple acid hydrolysis of the protein. (2) The levorotatory acids alone undergo racemization; the value of the rotation of the amino acids obtained from the racemized protein should have a higher dextro-rotation. (3) The dextrorotatory acids alone are racemized; the result should be the reverse of (2).

The observations made by us may be summarized as follows: (1) The amino acids obtained from gelatin which had been acted upon by 0.1 N alkali have a higher levorotation than those obtained in control experiments involving simple acid hydrolysis. (2) The amino acids obtained in experiments with 1.0 N alkali have a still higher levorotation. (3) The amino acids obtained in experiments with 3.0 N alkali have approximately the same rotation as the controls.

It should be added that the extent of hydrolysis of the gelatin (assuming that when hydrolysis is complete  $\frac{\text{amino nitrogen}}{\text{total nitrogen}} = 70$  per cent) in 48 hours by 0.1 N alkali was roughly 5 per cent, by 1.0 N alkali 45 per cent, and by 3.0 N alkali 75 per cent.

From these observations it may be concluded that in the case of 3.0 N alkali the hydrolysis proceeded so rapidly that racemization was prevented. With weaker alkali, since hydrolysis occurred more slowly, an opportunity was given for racemization. The increase in levorotation may therefore be interpreted in the sense of the racemization of the dextrorotatory amino acids in preference to that of the levorotatory.

The result of the action of alkali on gelatin is thus analogous to the action of alkali on ketopiperazines and therefore the data seem to point to the presence of ketopiperazines in the gelatin molecule. For a rigorous test of this possibility, it will be necessary to isolate the amino acids and to measure their optical activity.

In a qualitative way, however, the data obtained by Dakin<sup>2</sup> on

<sup>2</sup> Dakin, H. D., *J. Biol. Chem.*, 1912-13, xiii, 357. Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 263. Cf. Kossel, A., and Weiss, F., *Z. physiol. Chem.*, 1909, lix, 492; 1909, lx, 311; 1910, lxviii, 164; 1912, lxxviii, 402. Dakin, H. D., and Dale, H. H., *Biochem. J.*, 1919, xiii, 248.

the hydrolysis of racemized gelatin bear out our conclusions. This author found that leucine, aspartic acid, phenylalanine, histidine, and arginine were racemized, whereas proline, glutamic acid, and lysine remained active; alanine suffered partial racemiza-

TABLE I.

*Calculation of Rotations of Mixtures of Amino Acids Obtained by Hydrolysis of Gelatin and by Hydrolysis of Racemized Gelatin.*

Amino acid.	Per cent in gelatin.	$[\alpha]_D$ in excess of acid.	Rotation in hydrolysed gelatin.	Racemization.	Rotation in racemized gelatin.
	(1)	(2)	(3)	(4)	(5)
		<i>degrees</i>	<i>degrees</i>		<i>degrees</i>
Glycine.....	25.5				
Alanine.....	8.7	+10.3	+0.14	Partial.	+0.07
Leucine.....	7.1	+15.7	+0.18	Complete.	
Serine.....	0.4	+11.6	+0.01	(?)	+0.01
Phenylalanine....	1.4	-7.1	-0.02	Complete.	
Tyrosine.....	0.01	-8.5	0	(?)	
Proline.....	9.5	-48.6	-0.74	None.	-0.74
Hydroxyproline..	14.1	-49.2	-1.11	" (?)	-1.11
Aspartic acid....	3.4	+25.7	+0.14	Complete.	
Glutamic " ....	5.8	+30.8	+0.29	None.	+0.29
Histidine .....	0.9	+6.5	+0.01	Complete.	
Arginine .....	8.2	+20.8	+0.27	"	
Lysine.....	5.9	+17.5	+0.16	None.	+0.16
			-0.67		-1.32

The calculated rotations (Columns 3 and 5) are for solutions containing a mixture of amino acid equivalent to 1.000 gm. of gelatin in 25.0 cc. of acid, measured at 25°C. in 4.00 dm. tubes for the sodium D line. Column 1 gives the results of Dakin's analysis of gelatin. Column 2 gives the specific rotations of the amino acids compiled from the literature. Column 3 gives the calculated rotations for simple hydrolysis. The value  $-0.67^\circ$  is the algebraic sum. Column 4 gives the results of Dakin's study of the racemization of gelatin. While Dakin has not reported on the racemization of hydroxyproline, we have assumed that, in analogy with proline, it is not racemized. Column 5 gives the calculated rotations for racemized gelatin. 50 per cent racemization of alanine is assumed. The value  $-1.32^\circ$  is the algebraic sum.

tion. Assuming that the linkage of hydroxyproline in the gelatin molecule is similar to that of proline, the product of hydrolysis of racemized gelatin should have a higher levorotation than the product of simple acid hydrolysis.



To some extent our conclusions are substantiated quantitatively by a comparison of our results with the data obtained by Dakin, on one hand by the complete hydrolysis of gelatin,<sup>3</sup> on the other hand by the hydrolysis of racemized gelatin.<sup>2</sup> In Table I is given a calculated value of the rotation of a mixture of amino acids corresponding to the composition obtained by Dakin on hydrolysis of gelatin.<sup>4</sup> A mixture of these acids corresponding to a 4 per cent solution of gelatin should have a rotation of  $\alpha_D = -0.67^\circ$  in a 4 dm. tube. The acids obtained by the hydrolysis of racemized gelatin should have a rotation of  $\alpha_D = -1.32^\circ$ . The difference between the two values is  $0.65^\circ$ . In our experiments the rotation of the amino acids of unracemized gelatin was  $\alpha_{5461} = -0.36^\circ$ , and the rotation of the acids from the racemization experiments was  $\alpha_{5461} = -0.96^\circ$ , the difference between the two values being  $0.60^\circ$  (see Table V). This agreement is, in fact, above expectation. The results of the calculations are presented here only for the purpose of showing that the existing data do not contradict the assumption that on treatment of gelatin with dilute alkali for 48 hours the dextrorotatory acids suffer racemization in a higher degree.

An explanation should be given here for the discrepancies in the value of  $-0.67^\circ$  for the amino acids of unracemized gelatin given in Table I and the average experimental value of  $-0.36^\circ$  given for the controls in Table V. The reason lies in the fact that under the conditions of hydrolysis employed by us, namely hydrolysis with 5 N sulfuric acid at  $99.5^\circ\text{C}$ . for 24 hours, the amino acids undergo partial racemization. From Table II it is seen that at the maximum hydrolysis the product of hydrolysis of gelatin shows an amino nitrogen content (Van Slyke) of 70 per cent of the total nitrogen. A similar result was recently obtained by Greenberg and Burk.<sup>5</sup> When this degree of hydrolysis is obtained, the rotation of the product is determined by the conditions of hydrolysis.

<sup>3</sup> Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.

<sup>4</sup> It must be emphasized that the calculated values of the rotations of the hydrolysis products are for the sodium D line, while the rotation values determined experimentally are for the mercury green line 5461 Å. A comparison of the rotations of several amino acids for these two wave-lengths has shown that the differences are not of sufficient magnitude to invalidate the conclusions which we have drawn.

<sup>5</sup> Greenberg, D. M., and Burk, N. F., *J. Am. Chem. Soc.*, 1927, xlix, 275.

The maximum rotation ( $-0.92^\circ$ ) is obtained on hydrolysis with 3 N hydrochloric acid at  $125^\circ\text{C}$ . for 8 hours. These conditions were not adopted in the present work for the reason that difficulties were encountered in maintaining the temperature of  $125^\circ\text{C}$ . sufficiently constant. This difficulty, however, will be eliminated in our future work.

It also seems probable that the conditions for maximum racemization have not yet been established. On the basis of Dakin's analysis of gelatin an approximate calculation of the ionizable hydrogen in gelatin was made.<sup>6</sup> The equivalence of the alkali in our racemization experiments was approximately as follows: 0.1 N NaOH, 0.25 equivalents; 1.0 N NaOH, 2.5 equivalents; 3.0 N NaOH, 7.5 equivalents. In the experiments on ketopiperazines it was found that maximum racemization was obtained by the action of 1 equivalent of alkali in 0.1 N concentration.

#### SUMMARY.

1. The rotations and amino nitrogen ratios have been determined for the mixture of amino acids obtained from gelatin by acid hydrolysis under different conditions.

2. The rotations and amino nitrogen ratios have been determined for the mixture of amino acids obtained by acid hydrolysis of gelatin which was previously subjected to the action of alkali at  $25.0^\circ\text{C}$ .

3. It is shown that in gelatin racemization of some of the amino acids occurs on treatment with 0.1 N and with 1.0 N alkali, but not on treatment with 3.0 N alkali. The absence of racemization in the third case is explained by the fact that under these conditions the rate of hydrolysis is higher than the rate of racemization.

<sup>6</sup> This calculation was based on the ionizable hydrogen resulting from enolization of polypeptide or ketopiperazine linkages. Since the number of ionizable hydrogen atoms should be approximately the same in either case, the calculation is independent of any assumption of the presence of ketopiperazine structures. In regard to the ionization constants of ketopiperazines see Euler, H., and Pettersson, E., *Z. physiol. Chem.*, 1926, clviii, 7; Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, 1927, lxxii, 815.

4. These results, when compared with previous results on the racemization of ketopiperazines, indicate the possibility of the presence of ketopiperazines in the gelatin molecule.

5. The results are in harmony with the data of Dakin on the racemization of gelatin by alkali on one hand, and on the hydrolytic products of gelatin on the other.

#### EXPERIMENTAL.

In order to establish controls for the racemization experiments, an extended study was made of the effect of changes of conditions of hydrolysis with respect to concentration of acid, to temperature, and to duration of experiment upon the rotation of the hydrolytic product. The results are summarized in Table II.

The conditions chosen for the preliminary experiments were hydrolysis with 3 N  $\text{H}_2\text{SO}_4$  at  $99.5^\circ\text{C}$ . for 24 hours, since under these conditions the highest rotation, together with a comparatively high  $\frac{\text{amino nitrogen}}{\text{total nitrogen}}$  ratio, was obtained. However, although the results of racemization experiments were comparable to those obtained subsequently with 5 N  $\text{H}_2\text{SO}_4$ , the amino nitrogen ratio showed too much variation to warrant definite conclusions. Although the rotation of the control with 5 N  $\text{H}_2\text{SO}_4$  was comparatively low, our preliminary experiments with 3 N  $\text{H}_2\text{SO}_4$  had shown that the differences between this rotation and the rotations in the racemization experiments were large enough for our purpose.

Additional controls were run employing 5 N  $\text{H}_2\text{SO}_4$  and the quantities of  $\text{Na}_2\text{SO}_4$  equivalent to the alkali used in the racemizations. These data are recorded in Table IV.

The data on the racemization experiments are given in Table V.

*Acid Hydrolysis.*—Samples of gelatin<sup>7</sup> equivalent to 1.000 gm. of dry substance, weighed in 25.0 cc. calibrated flasks, were dissolved in the volume of standard acid required to fill the flasks to the mark. The solutions were transferred to Pyrex bomb

<sup>7</sup> The gelatin used in these experiments was kindly furnished by Dr. J. H. Northrop, who has developed a method of purification. We wish to express our appreciation of his courtesy. The same sample of gelatin was used throughout the investigation.

tubes, each containing 50.0 mg. of norit.<sup>\*</sup> The tubes were sealed and the contents mixed.

The hydrolyses at 99.5°C. were carried out in a large water bath with a rapid current of steam. The temperature was constant within  $\pm 0.3^\circ\text{C}$ . The hydrolyses at other temperatures were carried out in a large electric bomb tube furnace. The temperature as registered by thermometers was constant within  $\pm 1^\circ$ , but the difficulty of reproducing results, particularly the rotations, indicated that there was greater variation in different parts of the furnace.

TABLE II.  
*Rotations of Gelatin Hydrolyzed by Acid.*

Acid.	Temperature.	Time.	Amino nitrogen Total nitrogen	$\alpha_D^{25}$ corrected (4 dm. tube).
	$^\circ\text{C}$ .	hrs.	per cent	degrees
3.0 N HCl.	90	24	60.3	-3.46
3.0 " "	90	48	62.0	-2.67
3.0 " "	99.5	24	69.3	-0.74
3.0 " "	110	16	66.5	-1.44
3.0 " "	110	24	68.6	-0.96
3.0 " "	110	48	68.4	-0.42
3.0 " "	125	5	67.4	-1.56
3.0 " "	125	8	70.0	-0.92
3.0 " "	125	24	69.8	-0.40
2.0 " "	99.5	24	66.0	-1.40
3.0 " H <sub>2</sub> SO <sub>4</sub> .	99.5	24	64.4	-1.97
5.0 " "	99.5	24	67.9	-0.37

The tubes were opened and the contents were filtered through small filters into stoppered flasks. The rotations were measured at 25°C. for the wave-length 5461 Å. in 4.00 dm. open tubes. 2.00 cc. samples were taken for the determination of total nitrogen (Kjeldahl) and 1.00 cc. samples for amino nitrogen (micro Van Slyke).

\* A weighed quantity of 50.0 mg. of norit was used in each experiment in order to obtain perfectly colorless solutions for the polarimeter readings. The same sample of norit, which had been shaken thoroughly in a shaking machine to insure uniformity, was used throughout the investigation. Experiments without norit, which were decolorized after hydrolysis, proved that the results were not affected by its presence.

The results are recorded in Table II, each value being the mean of at least four experiments. In the calculation of the amino nitrogen/total nitrogen ratios, no corrections were made on the amino nitrogen values. The rotations in all experiments recorded in this paper were corrected to a concentration of 7.10 mg. of nitrogen per cc. The accuracy of the rotations is  $\pm 0.02^\circ$ .

TABLE III.  
*Hydrolysis of Gelatin by Alkali at 25.0°C.*

Alkali.	Time.	Total nitrogen per cc.	Amino nitrogen per cc.	$\frac{\text{Amino nitrogen}}{\text{Total nitrogen}}$
	<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
0.1 N	48	6.99	0.33	4.7
	48	7.04	0.32	4.5
	48	7.04	0.29	4.1
	Average.....			4.4
1.0 N	48	7.34	2.22	30.3
	48	7.13	2.31	32.4
	48	7.40	2.35	31.8
	Average.....			31.5
3.0 N	24	7.30	2.92	40.0
	24	6.96	2.92	42.0
	48	7.47	3.83	51.3
	96	7.30	3.70	50.7
	96	6.65	3.51	52.8
	Average.....			51.6

*Alkaline Hydrolysis.*—Samples of gelatin equivalent to 1.000 gm. of dry substance, weighed in 25.0 cc. calibrated flasks, were dissolved in the volume of standard alkali required to fill the flasks to the mark. The flasks were then placed in a thermostat at 25.0°C. and left for the required number of hours. The solutions were analyzed for total nitrogen and amino nitrogen. The data are recorded in Table III.

*Racemizations.*—Since the solutions in the racemization experiments contained sodium sulfate, resulting from the neutralization

of the alkali by  $\text{H}_2\text{SO}_4$ , it was necessary to run controls on acid hydrolyses containing equivalent quantities of this salt. Samples of gelatin equivalent to 1.000 gm. of dry substance were weighed

TABLE IV.  
*Control Experiments for Racemizations.*

Hydrolytic medium.	Total nitrogen per cc.	Amino nitrogen per cc.	Amino nitrogen Total nitrogen	$\alpha_{D445}$ corrected (4 dm. tube).
	mg.	mg.	per cent	degrees
5.0 N $\text{H}_2\text{SO}_4$ .	6.48	4.32	66.7	-0.36
	6.62	4.48	67.7	-0.34
	6.50	4.45	68.5	-0.40
	6.55	4.49	68.6	-0.38
Average.....			67.9	-0.37
5.0 N $\text{H}_2\text{SO}_4$	6.47	4.53	70.0	-0.40
+0.1705 gm.	6.63	4.64	69.9	-0.39
$\text{Na}_2\text{SO}_4$ .*	6.47	4.49	69.4	-0.36
	6.46	4.50	69.5	-0.37
Average.....			69.7	-0.38
5.0 N $\text{H}_2\text{SO}_4$	6.45	4.37	67.7	-0.39
+1.705 gm.	6.48	4.41	68.0	-0.36
$\text{Na}_2\text{SO}_4$ .†	6.39	4.41	69.1	-0.36
	6.69	4.52	67.6	-0.35
Average.....			68.1	-0.36
5.0 N $\text{H}_2\text{SO}_4$	6.52	4.33	66.4	-0.23
+5.115 gm.	6.55	4.48	68.4	-0.21
$\text{Na}_2\text{SO}_4$ ‡	6.85	4.63	67.6	-0.26
	6.41	4.36	68.1	-0.22
Average.....			67.6	-0.23

\* 0.1705 gm.  $\text{Na}_2\text{SO}_4 \equiv 24.0$  cc. 0.1 N NaOH.

† 1.705 gm.  $\text{Na}_2\text{SO}_4 \equiv 24.0$  cc. 1.0 N NaOH.

‡ 5.115 gm.  $\text{Na}_2\text{SO}_4 \equiv 24.0$  cc. 3.0 N NaOH.

into 25.0 cc. flasks. 12.5 cc. of 10.0 N  $\text{H}_2\text{SO}_4$  were added to each flask and the solutions were made up to 25.0 cc. with water. These solutions were introduced into bomb tubes containing 50.0 mg. of norit and the calculated quantities of solid sodium sulfate. The

TABLE V.  
*Racemization Experiments.*

Alkali.	Time.	Total nitrogen per cc.	Amino nitrogen per cc.	Amino nitrogen Total nitrogen	$\alpha_{D541}$ corrected (4 dm. tube).
	hrs.	mg.	mg.	per cent	degrees
0.1 N	24	6.12	4.27	69.8	-0.72
	24	6.45	4.52	70.1	-0.68
	24	6.42	4.38	68.2	-0.70
	24	6.64	4.44	66.9	-0.68
Average.....				68.7	-0.69
0.1 N	48	5.04	3.38	67.1	-0.77
	48	5.18	3.62	69.9	-0.81
	48	5.34	3.72	69.7	-0.80
Average.....				68.9	-0.79
Control (0.1 N).....				69.7	-0.38
1.0 N	24	6.52	4.61	70.8	-1.11
	24	6.41	4.44	69.2	-1.16
Average.....				70.0	-1.13
0.1 N	48	6.39	4.46	69.8	-1.01
	48	6.48	4.38	67.6	-1.03
	48	5.06	3.55	70.2	-0.95
	48	5.25	3.66	69.8	-0.91
	48	5.15	3.52	68.4	-0.92
Average.....				69.2	-0.96
Control (0.1 N).....				68.1	-0.36
3.0 N	24	5.37	3.70	69.0	-0.20
	24	5.29	3.60	68.1	-0.21
	48	5.10	3.54	69.5	-0.18
	96	5.18	3.68	71.0	-0.11
	96	4.73	3.33	70.4	-0.15
Control (3.0 N).....				67.6	-0.23

hydrolyses were then carried out at 99.5°C. for 24 hours. The data are recorded in Table IV and average values are given as controls in Table V.

The solutions for the racemizations were prepared as in the experiments on alkaline hydrolysis. After standing in the thermostat for the required time, they were neutralized exactly with standard sulfuric acid. The neutralized solutions were concentrated at 50°C. under reduced pressure to 10 cc. and were then transferred to calibrated 25.0 cc. flasks. To each flask were added 12.5 cc. of 10.0 N  $\text{H}_2\text{SO}_4$  and the solutions were made up to volume with water. These solutions in 5.0 N  $\text{H}_2\text{SO}_4$  were then sealed in bomb tubes with 50.0 mg. samples of norit and the hydrolyses were made at 99.5°C. for 24 hours. The rotations and analyses were made as in the case of the acid hydrolyses. The data are recorded in Table V.





# THE EFFECT OF IONIZATION UPON OPTICAL ROTATION.

## III. RELATIONS IN THE 2,5-ANHYDRO SUGAR ACIDS.

BY P. A. LEVENE AND LAWRENCE W. BASS.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

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It is known that the ion of an optically active electrolyte possesses a characteristic rotation which differs from that of the undissociated molecule.<sup>1</sup> The factors underlying this change in rotation are as yet not well known. On a previous occasion one of us expressed the view that the change in rotation was to a large extent due to the differences in the degree of distortion between the dissociated and the undissociated molecule.<sup>2</sup> The view then expressed was based on observations on gluconic and mannonic acids on one hand, and on 2,5-anhydromannonic (chitonic) and 2,5-anhydrosaccharic acids on the other. In the first two substances the direction of rotation changes on passing from the non-ionized to the ionized state, whereas in the other two, the direction of rotation remains the same and the numerical values of the two molecular species seem to differ but little from each other.

In connection with other work now in progress dealing with the problem of the influence of ionization on optical rotation, it seemed desirable to subject the view previously expressed to a more rigorous test and particularly to extend the observations to the 2,5-anhydrotetrahydroxyadipic acids. The present paper is a report on mannonic and 2,5-anhydromannonic (chitonic) acids and on mannosaccharic, 2,5-anhydromannosaccharic, and 2,5-

<sup>1</sup> Cf. Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, 1926, lxx, 243. Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, 1927, lxxii, 815.

<sup>2</sup> Levene, P. A., *J. Biol. Chem.*, 1924, lix, 135.

anhydrosaccharic acids. From the data (Table I)<sup>3</sup> it is clearly seen that the original assumption does not hold quantitatively; *i.e.*, that in the 2,5-anhydro acids the rotations of the undissociated acid and of its mono-ion are not identical. However, it is obvious that the assumption is qualitatively correct; namely, that

TABLE I.  
*Summary of Results.*

Acid.	pG <sub>1</sub> '	pG <sub>2</sub> '	pK <sub>1</sub> '	pK <sub>2</sub> '	[M <sub>u</sub> ]	[M <sub>m</sub> ]	[M <sub>d</sub> ]	[M <sub>u</sub> ] - [M <sub>m</sub> ]	[M <sub>m</sub> ] - [M <sub>d</sub> ]
Mannonic.....	3.36		3.36		-0.7	-24.2		23.5	
2,5-Anhydromannonic.....	2.89		2.89		+97.8	+81.1		16.7	
Mannosaccharic....	(3.1)	4.2	(3.1)	4.2	+29.2	+40	+6.7	11	33
2,5-Anhydromannosaccharic.....	3.01	3.67	2.92	3.76	+102.8	+112.4	+124.5	9.6	12.1
2,5-Anhydrosaccharic	2.01	4.76	2.01	4.76	+89.3	+102.0	+133.8	12.7	31.8

TABLE II.  
*Comparison of Change in Rotation with Change in Distance between Ionizable Groups.*

Acid.	$\frac{r_2}{r_1}$	$\frac{r_2}{r_1}$	$\frac{r_2}{r_1}$	$\frac{r_2}{r_1}$	$\frac{r_2}{r_1}$	[M <sub>u</sub> ] - [M <sub>m</sub> ]	[M <sub>m</sub> ] - [M <sub>d</sub> ]
	$\text{\AA.}$	$\text{\AA.}$	$\text{\AA.}$	$\text{\AA.}$	$\text{\AA.}$		
2,5-Anhydromannosaccharic.....	5.8	4.0	7.5	1.8	3.5	9.6	12.1
2,5-Anhydrosaccharic.....	4.6	1.2	7.0	3.4	5.8	12.7	31.8

the presence of the stabilizing ring has decreased the difference between the rotations of the different molecular species.

A more detailed comparison can be made in the case of the two 2,5-anhydrodicarboxylic acids. In a previous communication

<sup>3</sup> The data on mannosaccharic acid are not of a degree of accuracy to be compared with the data on the other acids, since neither the titrations nor rotations were satisfactory. However, since it was desired to compare the results of the 2,5-anhydrodicarboxylic acids with a dicarboxylic acid not containing a ring, the data on mannosaccharic acid have been included.

by Levene and Simms<sup>4</sup> a study was made of the relation between the dissociation constants of a dibasic acid and the distances between its ionizable groups, the latter being estimated from structural models. The values for 2,5-anhydrosaccharic and 2,5-anhydromannosaccharic acids are given in Table II, in which  $r_u$  is the distance between the ionizable groups in the undissociated molecule,  $r_m$  the distance between like charges in the mono-ion, and  $r_d$  the distance between like charges in the di-ion. From this table it is seen that there is a rough proportionality between the distances which the ionizable groups traverse in passing from one ionic species to another and the changes in rotation accompanying the transformations.

#### SUMMARY.

1. Titration-rotation data have been determined for mannonic, 2,5-anhydromannonic, mannosaccharic, 2,5-anhydromannosaccharic, and 2,5-anhydrosaccharic acids.

2. Using these data, accurate values of the rotations of the different molecular species have been calculated.

3. A comparison of these rotation values shows that the presence of a stabilizing ring decreases the change in rotation on passing from one molecular species to another.

4. A comparison of the data for the two 2,5-anhydrodicarboxylic acids shows that the change in rotation on passing from one molecular species to another is roughly proportional to the distance traversed by the ionizable groups during the transformation, as measured by means of structural models.

#### EXPERIMENTAL.

*Polarimetric and Potentiometric Measurements.*—For each substance a series of solutions of varying pH was prepared at 25°C. in 25.0 cc. flasks. The concentrations of these solutions were corrected on the basis of analyses.

The rotations were measured at 25°C. in 4.00 dm. open tubes (with glass plate covers) containing 22 cc. The readings were taken for the wave-length 5461 Å., with the exception of manno-

<sup>4</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxxiii, 351. Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1251.

saccharic acid solutions, for which the wave-length 5790.5 Å. was used because of the color of the solutions.

The pH measurements were made at 25.0°C. in water-jacketed hydrogen electrode cells, using the pH value 1.075 of 0.1000 N HCl as a standard and assuming constant liquid junction potential with saturated KCl.

*Calculations.*—The calculations from the titration data were made as in previous publications, except that hydrogen ion activities were not converted into concentrations in the equation

$$b' = \frac{b - a}{c} + \frac{H - OH}{c} \quad (1)$$

The overlapping constants of the dibasic acids were calculated by the method of Simms.<sup>5</sup>

The calculations from the rotation data of the degree of dissociation of the monobasic acids and of 2,5-anhydrosaccharic acid were made as in the preceding paper<sup>6</sup> by means of the relation

$$\gamma = \frac{[M_u] - [M]}{[M_u] - [M_m]} \quad (2)$$

In mannosaccharic and 2,5-anhydromannosaccharic acids the fractions present as unionized substance, mono-ion, and di-ion (designated as  $u$ ,  $m$ , and  $d$ , respectively) were calculated from the following equations which are derived from the classical equations for the ionization of a divalent acid.<sup>7</sup>

$$\frac{1}{K_2'} = \frac{1}{G_1'} + \frac{1}{G_2'}, \text{ or } K_2' = \frac{G_1' G_2'}{G_1' + G_2'} \quad (3)$$

$$d = \frac{b' K_2'}{H + 2K_2'} \quad (4)$$

$$m = b' - 2d \quad (5)$$

$$u = 1 - b' + d \quad (6)$$

<sup>5</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

<sup>6</sup> Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, 1927, lxxii, 815.

<sup>7</sup> Equations 4, 5, and 6 follow from  $u + m + d = 1$ ,  $b' = m + 2d$ ,  $K_2' = H \frac{d}{m}$ .

From the experimental values of  $[M_u]$  and  $[M_d]$  and the calculated values of  $u$ ,  $m$ , and  $d$ , the value of  $[M_m]$  was found from the equation

$$[M_m] = \frac{[M] - d[M_d] - u[M_u]}{m} \quad (7)$$

TABLE III.  
*Mannonic Acid. (0.1000 Molar.)*

pH	$\frac{b-a}{c}$	Titration.		$\alpha$	[M]	Rotation.	
		$b' = \gamma$	pG'			$\gamma$	pG'
1.63	-0.200	0.034		-0.03	-0.7		
2.22	0	0.060	(3.42)	-0.06	-1.5	0.034	(3.67)
2.26	0	0.055	(3.49)	-0.08	-2.0	0.055	(3.49)
2.93	0.250	0.262	3.37	-0.27	-6.7	0.255	3.39
3.38	0.500	0.504	3.37	-0.50	-12.5	0.502	3.38
3.83	0.750	0.751	3.35	-0.74	-18.5	0.758	3.33
5.94	1.000			-0.96	-24.0		
12.04	1.200			-0.97	-24.2		
Average.....			3.36				3.37

TABLE IV.  
*2,5-Anhydromannonic Acid. (0.525 Molar.)*

pH	$\frac{b-a}{c}$	Titration.		$\alpha$	[M]	$[M_m]$	Rotation.	
		$b' = \gamma$	pG'				$\gamma$	pG'
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
0.60	-0.428			+20.54	+97.8			
1.55	0.048	0.102	(2.81)	+20.28	+96.6		0.072	(2.66)
2.44	0.263	0.270	2.87	+19.57	+93.2	+80.8	0.276	2.85
2.94	0.525	0.527	2.89	+18.68	+89.0	+81.1	0.527	2.89
3.47	0.788	0.789	2.90	+17.89	+85.3	+81.3	0.749	3.00
6.56	1.000	1.000		+17.12	+81.5			
Average.....			2.89			+81.1		2.91

*Mannonic Acid.*—The mannonic lactone used in these experiments gave the following analysis.

No. 962.

Calculated for  $C_6H_{10}O_6$ . C 40.44, H 5.66.  
Found. " 40.38, " 5.81.

In the preparation of the solutions, individual samples of the lactone were weighed in 25.0 cc. flasks. To each sample 1.200 equivalents of N alkali and sufficient water to make the total volume approximately 15 cc. were added. These solutions were then allowed to stand tightly stoppered overnight to insure complete hydrolysis of the lactone. The resulting solutions of the sodium salt were made up with varying quantities of acid. The polarimetric and potentiometric readings were made as rapidly as

TABLE V.  
*Titration Data of Mannosaccharic Acid. (0.0100 Molar.)*

Series.	pH	$\frac{b-a}{c}$	b	Calculation of $pG_2'$ .		
				$(pG_1' = 3.1)$	$\gamma_2$	$pG_2'$
A	1.59	-0.20				
"	1.99	0				
B	2.47	0.25	0.59			
A	2.57	0.35	0.62			
B	2.74	0.50	0.68			
A	3.04	0.65	0.74			
B	3.14	0.75	0.82			
"	3.51	1.00	1.03	0.72	0.31	(3.89)
A	3.59	1.00	1.03	0.75	0.28	(3.99)
B	3.90	1.25	1.26	0.86	0.40	4.08
A	4.15	1.35	1.36	0.92	0.44	4.25
B	4.29	1.50	1.50	0.94	0.56	4.19
A	4.62	1.65	1.65	0.97	0.68	4.29
B	4.74	1.75	1.75	0.98	0.77	4.22
A	6.78	2.00				
"	10.88	2.20				
Average.....						4.2

possible on each solution as soon as it had been prepared, in order to avoid errors due to lactone formation.

The titration-rotation data are recorded in Table III. The values used for the calculation of  $\gamma$  are  $[M_u] = -0.7$  and  $[M_m] = -24.2$ .

*2,5-Anhydromannonic Acid (Chitonic Acid).*—The calcium chitonate used in these experiments gave the following analysis (calculated as anhydrous salt).

No. 918.

Calculated for  $C_6H_{10}O_5$ , ca. C 36.54, H 4.56, Ca 10.15.

Found. " 36.49, " 4.47, " 10.20.

The titration-rotation data are recorded in Table IV. The value of  $[M_m]$  at a higher pH could not be determined experimentally because of the precipitation of calcium hydroxide. Hence a more accurate value of  $[M_m]$  was calculated by Equation 2, using  $[M_u] = +97.8$  and the titration values of  $\gamma$ . The calculated

TABLE VI.  
*Rotation Data of Mannosaccharic Acid. (0.1000 Molar.)*

$\frac{b-a}{c}$	$\alpha$	[M]	pH	$b'$	$u$	$m$	$d$	$[M_m]$
-0.200	+1.17	+29.2						
0.250	+1.28	+32.0	2.47	0.283	0.722	0.273	0.005	+40.0
0.500	+1.53	+38.2	2.74	0.518	0.498	0.486	0.016	(+48.6)
0.500	+1.43	+35.7	2.74	0.518	0.498	0.486	0.016	+43.4
0.750	+1.39	+34.7	3.14	0.757	0.296	0.651	0.053	+39.7
1.000	+1.33	+33.2	3.59	1.00 <sub>3</sub>	0.155	0.687	0.158	+40.3
1.25	+1.01	+25.2	3.90	1.26 <sub>1</sub>	0.044	0.651	0.305	(+33.7)
1.50	+0.70	+17.5	4.29	1.50 <sub>0</sub>	0.024	0.472	0.524	(+28.2)
1.75	+0.41	+10.2	4.74	1.75 <sub>0</sub>	0.009	0.232	0.759	(+20.7)
2.00	+0.32	+8.0						
2.20	+0.27	+6.7						
Average.....								+40

values of  $[M_m]$  are given in Column 7, the average being +81.1. Employing  $[M_u] = +97.8$  and  $[M_m] = +81.1$ , the values of  $\gamma$  were then calculated.

*Mannosaccharic Acid.*<sup>3</sup>—The mannosaccharic lactone used in these experiments gave the following analysis.

No. 977.

Calculated for  $C_6H_8O_6$ . C 41.40, H 3.48.

Found. " 41.61, " 3.55.

Preliminary experiments showed that the acid could not be titrated in 0.1 M solution, but that fair readings could be obtained in 0.01 M solution.

A sample of the lactone equivalent to 50.0 cc. of 0.0200 M solution was weighed into a calibrated flask, 2.20 equivalents of alkali were



added, and the solution was made up to 50.0 cc. After standing tightly stoppered overnight to insure complete hydrolysis of the

TABLE VII.

*Titration Data of 2,5-Anhydromannosaccharic Acid. (0.1000 Molar.)*

pH	$\frac{b-a}{c}$	$b'$	Calculation of $pG_1'$			Calculation of $pG_2'$		
			$\gamma_2$ ( $pG_2' = 3.66$ )	$\gamma_1$	$pG_1'$	$\gamma_1$ ( $pG_1' = 3.01$ )	$\gamma_2$	$pG_2'$
1.99	0	0.102						
2.47	0.250	0.284	0.061	0.223	3.01	0.228	0.056	3.69
2.83	0.500	0.515	0.129	0.386	3.03	0.404	0.111	(3.73)
3.07	0.750	0.758	0.212	0.546	2.99	0.552	0.206	3.66
3.34	1.000	1.004	0.324	0.680	3.01	0.687	0.317	3.67
3.59	1.250	1.253	0.460	0.793	3.01	0.796	0.457	3.66
3.87	1.500	1.501	0.618	0.883	2.99	0.881	0.620	3.66
4.27	1.750	1.751	0.802	0.949	3.01	0.949	0.802	3.66
8.94	2.000							
12.05	2.250							
Average.....					3.01			3.67

TABLE VIII.

*Rotation Data of 2,5-Anhydromannosaccharic Acid. (0.1000 Molar.)*

pH (1)	$\alpha$ (2)	[M] (3)	$u$ (4)	$m$ (5)	$d$ (6)	[M <sub>m</sub> ] (7)	[M <sub>u</sub> ] (8)
1.99	+4.14	+103.6	0.900	0.098	0.002	(+108.2)	+102.5
2.47	+4.23	+105.7	0.729	0.258	0.013	+112.4	+103.0
2.83	+4.30	+107.5	0.535	0.415	0.050	+111.4	+102.2
3.07	+4.43	+110.7	0.356	0.530	0.114	+112.4	+103.6
3.34	+4.54	+113.6	0.214	0.568	0.218	+113.4	(+105.0)
3.59	+4.63	+115.7	0.110	0.527	0.363	+112.3	+102.7
3.87	+4.70	+117.4	0.041	0.417	0.542	(+109.9)	
4.27	+4.83	+120.7	0.008	0.233	0.759	(+109.9)	
8.94	+4.98	+124.6					
12.05	+4.97	+124.2					
Average.....						+112.4	+102.8

lactone, 5.00 cc. samples were transferred to 10.00 cc. flasks, and this series of solutions was made up with varying quantities of acid. The pH of each 0.0100 M solution was determined as rapidly as

possible to avoid lactone formation. A second series of titrations was run as a check. The data are recorded in Table V. The value of  $pG_1'$  was taken as 3.1.

For the rotation determinations, samples of the lactone equivalent to 0.1000 M solutions were weighed into 25.0 cc. flasks, 2.20 equivalents of alkali were added to each sample, and then sufficient water to make the volume about 15 cc. After standing tightly stoppered overnight to insure complete hydrolysis, the solutions were made up to 25.0 cc. with the required volumes of acid and water. The rotations of the resulting solutions, which were slightly colored, were read as rapidly as possible as soon as each had been prepared. The data are recorded in Table VI.

Since it was not possible to determine the pH of these solutions experimentally, the pH values of the 0.0100 M solutions at the same values of  $\frac{b-a}{c}$  were used.<sup>8</sup> In the calculation of  $[M_m]$  (last column), only the values at  $\frac{b-a}{c} = 0.250, 0.500, 0.750, \text{ and } 1.000$  are in agreement with the value to be expected from an inspection of the rotation data.

*2,5-Anhydromannosaccharic Acid.*—The acid used in these experiments gave the following analysis.

No. 648.

Calculated for  $C_6H_8O_7$ . C 37.50, H 4.20.

Found. " 37.39, " 4.29.

The titration data are recorded in Table VII. In Table VIII are given the rotation data and the calculated values of  $u$ ,  $m$ , and  $d$ . Using the experimental value  $[M_d] = +124.5$  and an estimated value  $[M_u] = +103.0$ ,  $[M_m]$  was calculated, the values being given in Column 7, Table VIII. Using the best average  $[M_m] = +112.4$ , a more accurate  $[M_u]$  was calculated (Column 8).

*2,5-Anhydrosaccharic Acid.*—The acid used in these experiments gave the following analysis (calculated as anhydrous acid).

No. 693.

Calculated for  $C_6H_8O_7$ . C 37.50, H 4.20.

Found. " 37.30, " 4.16.

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<sup>8</sup> This procedure, which is, of course, a rough approximation, was adopted in order to obtain a value for  $[M_m]$ .

TABLE IX.  
*2,5-Anhydrosaccharic Acid.*

pH (1)	$\frac{b-a}{c}$ (2)	b' (3)	Titration.				$\alpha$ (8)	[M] (9)	[M <sub>m</sub> ] (10)	Rotation.				
			$\gamma_1$ (4)	$\gamma_2$ (5)	pG <sub>1</sub> ' (6)	pG <sub>2</sub> ' (7)				$\gamma_1$ (11)	$\gamma_2$ (12)	pG <sub>1</sub> ' (13)	pG <sub>2</sub> ' (14)	
0.204 molar.														
0.72	-0.983						+7.27	+89.3						
1.72	0.246	0.340	0.340			2.01	+7.66	+94.1	+103.5	0.378		1.94		
2.07	0.491	0.533	0.533			2.01	+7.81	+95.9	+100.3	0.520		2.04		
2.49	0.737	0.753	0.753			2.00	+8.09	+99.4	+101.7	0.720		2.09		
3.27	0.986	0.986					+8.50	+104.4						
0.102 molar.														
4.25	1.229	1.229	1.000	0.229			+4.50	+110.5	+103.6	1.000	0.267		4.68	
4.71	1.474	1.474	1.000	0.474		4.78	+4.81	+118.1	+100.6	1.000	0.506		4.70	
5.15	1.720	1.720	1.000	0.720		4.75	+5.08	+124.9	+102.1	1.000	0.720		4.75	
6.30	1.965	1.965					+5.36	+131.6						
12.04	2.210						+5.45	+133.8						
Average.....			2.01			4.76			+102.0			2.02	4.71	

The titration-rotation data are recorded in Table IX. A stronger concentration was used in the first series of experiments because of the low value of  $[M_u] - [M_m]$  as compared with  $[M_m] - [M_d]$ . The experimental values  $[M_u] = +89.3$  and  $[M_d] = +133.8$  were used in the calculations. Because of slight overlapping of  $pG_1'$  and  $pG_2'$  a good value of  $[M_m]$  could not be obtained experimentally. A more accurate evaluation was made by Equation 2, using the titration values of  $\gamma_1$  and  $\gamma_2$ . The calculated values of  $[M_m]$  are given in Column 10. The average value  $+102.0$  was then used in calculating  $\gamma_1$  and  $\gamma_2$  from rotation data.



## TOTAL SUGAR OF BLOOD AND URINE.

By MARK R. EVERETT, HAROLD A. SHOEMAKER, AND  
FAY SHEPPARD.

*(From the Department of Biochemistry and Pharmacology, University of  
Oklahoma Medical School, Norman.)*

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The determination of total sugar of urine and deproteinized blood filtrate is a matter of recent date. In fact, the method of Folin and Berglund (1) is the only attempt worthy of detailed consideration, earlier work being of a scattered and doubtful nature. These authors used 3 drops of 10 per cent hydrochloric acid solution for the hydrolysis of the combined sugar in 2 cc. of Folin-Wu blood filtrate, and 1 cc. of the same acid for 10 cc. of Folin-Berglund urine filtrate. The mixtures were heated for 1½ hours on the water bath, then cooled, and neutralized with equivalent sodium hydroxide solution. Folin and Berglund (2) employed this method extensively and published 150 analyses for total sugar in blood filtrates. Of these analyses 63 per cent were lower than the free sugar analyses in the same filtrates. Similarly conducted analyses on urine showed 5 per cent of the 140 analyses for total sugar to be lower than the free sugar.

One would naturally suspect the accuracy of a method which yielded such results, yet Folin and Berglund arrived at the following conclusions, in their second paper.

"In a later section we call attention to the diminution of the blood sugar obtained after hydrolysis. A similar diminution has in certain cases been found in urine also. . . . We have no explanation to offer, but are certain that the finding does not depend on analytical errors."

Again, in the later section referred to, they said:

"From the sugar obtained after hydrolysis it has become clear to us that the subject under discussion, . . . is further obscured by the uncertainty as to the nature of a part of the blood sugar. There are present

in the blood, and, we believe, also in the urine, reducing substances which disappear during the hydrolysis. . . . These substances are more prominent in the plasma . . . . The increases produced by hydrolysis derived chiefly from the corpuscles may be due wholly, or in part, to glycogen . . . though we have not been able to prove it. Oyster glycogen added to whole blood immediately before precipitating with sodium tungstate and sulfuric acid is precipitated so completely that not a trace goes into the filtrate."

Recently we attempted to use the method of these authors in certain metabolic experiments and obtained similar low results for total sugar. We noted an additional disadvantage. The blue colors resulting from the application of the Folin-Wu method to the hydrolysates of blood filtrate faded so rapidly that accurate readings were impossible. The analysis by the Folin-Berglund method for total sugar of twenty-two urines from a normal, fasting man showed that, while the blue colors were more permanent, 73 per cent of these values were lower than those for the free sugar. When Sumner's method was substituted for the Folin-Wu method, in the sugar determinations, only 46 per cent gave lower total sugar figures. In order to carry out the Sumner determinations, we used urine filtrates from bone-black, conducted the hydrolyses according to Folin and Berglund, and analyzed the hydrolysates by Sumner's method (3).

Low values and rapid fading of the blue color are not the only defects of the Folin-Berglund procedure, because the lack of careful neutralization of the hydrolysates of blood filtrate render impossible the use of new sugar reagents of low alkalinity. A slight variation in the size of the drops of acid or alkali added by Folin and Berglund to the blood filtrates is enough to destroy entirely the alkalinity of the new reagent of Folin (4).

Everett and Shoemaker (5) have reported a new procedure for the determination of total sugar applicable to most of the common, modern, colorimetric methods. In the following pages is recorded the experimental work which led to the development of this method.

#### EXPERIMENTAL.

In these experiments we employed the colorimetric methods of Folin and Wu (6), Benedict (7), Sumner (3), and Folin (4). The proportionality of color to sugar concentration is quite satisfactory

for all of these except the Folin-Wu method. In order to make our results more comparative we applied to the Folin-Wu values the corrections of Oser and Karr (8) which were applicable to our reagents as long as readings were kept well within the limits suggested by the authors. It is almost impossible to correct for colors which are too divergent and in such cases we repeated the analysis. The sugar standards were prepared from anhydrous dextrose, c.p. The several standards were ascertained to be proportional to each other and were preserved with benzoic acid or toluene, as the conditions indicated.

In the last two columns of Table I are recorded typical analyses of blood filtrate for total sugar by the Folin-Berglund method.

TABLE I.  
*Relative Rates of Fading.*

Time after completion of analysis.	0.2 mg. glucose standard.				Blood filtrate.	
	NaCl		Na <sub>2</sub> SO <sub>4</sub>		HCl-NaOH	H <sub>2</sub> SO <sub>4</sub> -NaOH
	Folin-Wu.	Folin.	Folin-Wu.	Folin.	Folin-Wu.	Folin-Wu.
<i>min.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
10	-29		+2		86	98
15		-10		-2		
20	-36		-1	-4		
25			+2			
30	-43	-11			77	100
45	-48			-2	75	101

Readings made after 10 minutes indicated that the free sugar of this particular blood filtrate was 90 mg. per cent, and after 30 minutes, 89 mg.

The substitution of an equivalent amount of sulfuric acid for the hydrochloric acid of Folin and Berglund gives both greatly increased total sugar values and more permanent colors. The other experiments recorded in the table show clearly that it is the sodium chloride, formed by the neutralization of the hydrochloric acid, which is responsible for the low values and rapid fading. By use of 2 cc. of 0.01 per cent dextrose solution and 0.2 cc. of 2.78 N sodium chloride solution (an amount roughly equivalent to that present in the hydrolysates) the same results are secured,



while the addition of equivalent amounts of sodium sulfate has very little effect. The new Folin method is not as susceptible to the action of sodium chloride as the older Folin-Wu method.

If sodium chloride were added to the standard also, the effect on the analytical result would be minimized. Folin and Berglund do not indicate that this was done and we feel that even though the step were included, their procedure would still be quite inaccurate

TABLE II.  
*Effects of Salts.*

The results are expressed as per cent deviation from the theoretical values.

Method.	LiCl	NaCl	NaCl after heat- ing.	KCl	RbCl	CsCl
Folin-Wu.....	-53	-45	-32	-41	-43	-39
Folin.....	-36	-23	-12	-17	-17	-16

Method.	Li <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub> after heating.	K <sub>2</sub> SO <sub>4</sub>
Folin-Wu.....	+3	+8	-2	+4
Folin.....	-13	-10	0	-4
Benedict.....		-7		-8

Method.	Na citrate.	Na citrate after heat- ing.	Na <sub>2</sub> SiO <sub>3</sub>			
			0.2 N	0.8 N	0.8 N after heat- ing.	2.78 N
Folin-Wu.....	-37	+10	-6	-21	-7	-38
Folin.....	-58		-8	-31	-4	-40
Benedict.....	+3			-8		
Sumner.....	-6			0		

because of the probable unequal rate of fading in standard and unknown. It would be much better to eliminate from the latter the rapid fading and thus avoid the decrease of color intensity. Table II shows that all chlorides have this depressing effect on color formation in the Folin-Wu method and a similar but smaller effect in the Folin method.

In the experiments recorded in Table II 0.2 cc. of a 2.78 N

solution of the c.p. salt was added to the Folin-Wu sugar tube before the addition of the copper or dinitrosalicylic acid reagent. 2 cc. of 0.01 per cent dextrose solution were used for the copper methods and 1 cc. of 0.05 per cent for the Sumner method. All readings were made 35 minutes after the completion of the analysis, in order to provide standard conditions of fading. The addition of the salt solution was balanced in the standards by an equal amount of water. The figures reported are averages of a number of determinations and are expressed in terms of per cent deviation from the theoretical values. Chlorides impart a decided greenish cast to the blue color developed in the Folin-Wu and Folin methods, making comparison difficult.

TABLE III.

*Effect of Addition of Acid and Alkali.*

The results are expressed as per cent deviation from the theoretical values.

Method.	HCl and NaOH	HCl and KOH	H <sub>2</sub> SO <sub>4</sub> and NaOH	H <sub>2</sub> SO <sub>4</sub> and KOH	H <sub>2</sub> SO <sub>4</sub> - NaOH vs. Na <sub>2</sub> SO <sub>4</sub> standard.	HCl- NaOH vs. NaCl standard.
Folin-Wu.....	-35		-8	+2	-9	-17
Folin.....	-40		-28	+1	-24	-28
Benedict.....	-2	-2	+2	-1		
Sumner.....	-3	+2	+2	0		

The various alkali chlorides may be arranged in the order of their depressing effect. This is the same order as that found by Michaelis and Krüger (9) for the depression of the pH of buffer solutions. The same authors found the chloride ion to be more depressant than the sulfate ion. It would be of interest to know whether the chlorides produce a similar effect on the buffers of the alkaline copper mixtures. In 1922 Cooper and Walker (10), using MacLean's method, found that chlorides, bromides, iodides, and citrates inhibited the reduction of the copper reagent, while sulfates and phosphates did not. They thought that compounds were formed between these ions and sugar. However, in the Folin-Wu and Folin methods the addition of sodium chloride, after the copper reduction has taken place, still produces a marked depression of color. Hence the mode of action is uncertain.

Chlorides have practically no effect upon the Benedict or Sumner methods. We include no figures for these methods in this table, but the results in Table III prove the point.

Our results show that sulfates have relatively little effect on the copper methods and none on Sumner's method. The Folin and Benedict methods seem to be slightly affected by sodium sulfate, the Folin method less so by potassium sulfate. Moreover, there is no perceptible fading during the reading of any of these samples. Sodium citrate has a marked inhibitory action on the color production in the Folin-Wu and Folin methods, an effect which is chiefly on the reduction of the copper. The Benedict method is little affected for obvious reasons.

We are more interested in the action of sodium silicate, an inhibitory action on color production in the Folin and Folin-Wu methods fully equal to that of the chlorides. Again the effect seems to be chiefly on the stage of copper reduction and is more marked in the new Folin method. We shall return to the silicate effect later, but it may be remarked in passing that neither here nor in the case of citrates is there any fading comparable to that produced by the chlorides.

The greater effect of chlorides, citrates, and silicates on the Folin and Folin-Wu reagents might be due to the lower sodium content, greater phosphate content, or the presence of molybdate in these reagents. The Benedict reagents are the only ones which contain chlorides or citrates. We may conclude from our data that sulfuric acid may be present in the hydrolysates to be analyzed by any of the four methods, while hydrochloric acid may be present only in those to be analyzed by the Benedict or Sumner methods.

We next tried the effects of adding acid and alkali to the standard dextrose solutions. The results are recorded in Table III, in terms of per cent of deviation from the theoretical values.

In this case the analytical samples were taken from solutions consisting of 8 cc. of the standard sugar solution, 1 cc. of 2.6 N acid, and 1 cc. of carefully adjusted, equivalent alkali. By measuring the acid and alkali with the same Ostwald-Folin pipette we secured very careful neutralization, less than 1 drop of 0.1 N alkali or acid being necessary to turn the color of phenolphthalein when added to 2 cc. of the mixture. The dilutions are taken into account in the results reported, the latter being averages of several

determinations. The salt standards were prepared as indicated previously.

The data show clearly that the mere substitution of sulfuric acid for hydrochloric acid is not sufficient to prevent all inhibition of color production in the Folin-Wu and Folin methods. The last two columns of Table III show that we have not only the salt effects of sulfates and chlorides, but also an additional inhibiting factor. Suspecting that silicates in the sodium hydroxide were the cause, we prepared an equivalent potassium hydroxide solution and kept it in a paraffined bottle. The choice of potassium hydroxide was made because of its relative cheapness, the less

TABLE IV.  
*Glucose Equivalents in Per Cent of Glucose Reduction.*

Sugar.	(Rowe and Wiener) Folin-Wu.	(Greenwald, <i>et al.</i> ) Folin-Wu.	Folin-Wu.	Folin.	Benedict.	Sumner.	(Greenwald, <i>et al.</i> ) Sumner.
Levulose (Eimer and Amend).....	90	91	94	102	115	98	100
Galactose (Eastman).....	77	75	75	49	68	93	95
Mannose (Eimer and Amend).....	55	58	59	46	80	97	76
Xylose (Pfanstiehl).....		94	101	84	112	114	114
Arabinose (Pfanstiehl).....		80	82	58	77	116	117
Lactose.H <sub>2</sub> O (Pfanstiehl).....	45	45	43	27	59	77	70
Maltose.H <sub>2</sub> O (Eimer and Amend)	40	40	40	44	67	75	70

pronounced inhibitory action of its salts (*cf.* Table II), and its slight solvent action on glass. The use of this alkali was fully justified, as the results in the second and fourth columns of Table III illustrate.

We may conclude that the low values secured by the Folin-Berglund method are due to two factors; namely, the effects of sodium chloride, which is formed during the neutralization, and of silicates present in the alkali used for the neutralization. Therefore, we recommend the use of 2.6 N sulfuric acid and silicate-free potassium hydroxide as a general procedure for the determination of total sugar by colorimetric methods.

In order to test the method by hydrolyzing some simple known

sugar compounds, it was necessary to have glucose equivalents of other sugars. In the literature, one can find equivalents for the Folin-Wu method, but not for the other methods. The equivalents of Greenwald, *et al.* (11), which are recorded in the last column of Table IV, were determined by an earlier modification of Sumner's method which has now been supplanted. We have also included, for purposes of comparison, the recently determined equivalents of Rowe and Wiener (12). The same concentrations of sugar solutions were used as in previous experiments, except in the case of lactose, maltose, mannose, and galactose, where 2 cc. of 0.02 per cent solutions were used for the copper methods. The figures reported have a probable limit of error of 1 to 2 per cent and are averages of a number of determinations.

Our Folin-Wu equivalents agree quite well with those of previous investigators, except in the case of xylose. Our equivalent for this sugar, by the Sumner method, agrees with that of Greenwald, *et al.* Our sample of levulose, which we frankly suspect of being impure, reduced a little more copper than expected. We repeated the comparison of these two sugars many times, with the same results. Hence these differences probably depend upon the purity of the several sugar samples in question. The sources of our sugars are given in the table.

There are no published data with which to compare the values for the new Folin and Benedict methods. In the Folin method all equivalents except those of levulose and maltose are greatly reduced, an interesting theoretical consideration. A word of caution is necessary. One must use fresh Folin copper reagent to determine these equivalents, older solutions being likely to yield entirely different results. Benedict's method gives higher equivalents for all sugars except galactose and arabinose.

Probably there can be no fair comparison between our Sumner equivalents and those of Greenwald, *et al.*, for reasons already mentioned. We get a much higher mannose equivalent with the newer Sumner reagent and we believe this figure to be correct because of the apparently satisfactory Folin-Wu equivalent for the same sugar. The two disaccharides were found to have higher and levulose a slightly lower equivalent in our analyses.

The equivalents of five of these sugars were determined by the several methods upon mixtures containing acid and alkali, pre-

pared as described for the experiments reported in Table III. From the data secured, it was apparent that the presence of the potassium sulfate from the neutralization of the hydrolysates would have no effect upon the glucose equivalents as recorded in Table IV.

It was also necessary to determine the amount of destruction of the various monosaccharides under the conditions of the hydrolysis in the total sugar method. Accordingly, we heated 8 cc. of each sugar standard on the water bath for  $1\frac{1}{4}$  hours with exactly 1 cc. of 2.6 *N* acid. (We had decided temporarily to employ the conditions of Folin and Berglund.) At the end of that time the mixtures were cooled and neutralized with exactly 1 cc. of equivalent, silicate-free

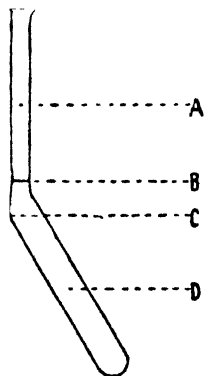


FIG. 1. A, inside diameter 7.5 mm.; B, calibration for 10 cc.; C, 9 cc. of hydrolysate to reach this level; D, inside diameter 12 mm. The tube is bent at an angle of  $150^\circ$ .

potassium hydroxide solution. In order to dilute the entire mixture to a volume of exactly 10 cc. some form of volumetric flask was desirable. We finally adopted a specially designed Pyrex flask as the most convenient receptacle. The diagram (Fig. 1) shows that this tube<sup>1</sup> allows easy mixing of its contents, both before hydrolysis and after neutralization, and prevents undue evaporation.

In order to minimize errors, portions of these acid-treated sugar samples were compared with standards containing the same

<sup>1</sup> Pyrex tubes of this design, together with the necessary 8 cc. pipettes, may be purchased from the Will Corporation, Rochester, N. Y.

amount of acid and alkali, but which had not been heated. The resulting data are recorded in Table V.

We are chiefly interested in the potassium hydroxide values. It is apparent that under the specified conditions there is no appreciable loss of dextrose. In fact, levulose appears to be the only common hexose which is partially destroyed, the Folin-Wu and Folin methods being the most sensitive to this change. Some

TABLE V.  
*Destruction of Monosaccharides by Heating with Acids.*

Sugar.	Acid.	Alkali.	Folin-Wu.	Folin.	Benedict.	Sumner.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Glucose.	H <sub>2</sub> SO <sub>4</sub>	NaOH	-1	+1	0	-2
"	"	KOH	-1½	-1½	+1	-3
Levulose.	H <sub>2</sub> SO <sub>4</sub>	NaOH	-9	-10		-4½
"	"	KOH	-12	-13	-2½	-5
"	HCl	"				-17
Galactose.	H <sub>2</sub> SO <sub>4</sub>	NaOH	+5	+7		-3
"	"	KOH	+3	+6	+1½	+5
Mannose.	H <sub>2</sub> SO <sub>4</sub>	KOH	+2	-2	-1½	-3
"	HCl	"			-3	
Arabinose.	H <sub>2</sub> SO <sub>4</sub>	KOH	-7	-12	-6	-1
"	HCl	"			-7	
Xylose.	H <sub>2</sub> SO <sub>4</sub>	KOH	-2	-5½	-1½	+2
"	HCl	"			-2½	

The figures for the pentoses are individual determinations, not averages of a number of analyses.

arabinose is also lost by heating with acid. As a rule, more sugar is lost when hydrochloric acid is used. Similar decreases have been demonstrated by others, but whether actual destruction or polymerization takes place is uncertain. It will be noticed that there are small, but undoubted, increases in the apparent galactose values after the hydrolytic treatment. These various changes must be included in calculating the rate of hydrolysis of known sugar compounds.

We have now proven every step of the new total sugar procedure. There remains a possible disturbing factor which it is very difficult to ascertain completely. By this is meant the probability that the particular isomers of the monosaccharides, liberated during the hydrolysis, react differently to the sugar reagents in comparison with the more stable forms used in our investigation. In fact, the figures recorded in Table VI suggest this circumstance.

Pure disaccharides were subjected to the new total sugar procedure in order to measure the degree of hydrolysis obtainable under the

TABLE VI.  
*Calculated Degree of Hydrolysis with Final Procedure.*

Sugar.	Acid.	Alkali.	Folin-Wu.	Folin.	Benedict.	Sumner.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Sucrose.	H <sub>2</sub> SO <sub>4</sub>	NaOH	108		101.5	97.5
"	"	KOH	108	103.5	98	96.5
Factors.			1.935	2.01	2.225	1.015
Lactose. H <sub>2</sub> O	H <sub>2</sub> SO <sub>4</sub>	NaOH	86	86	92	95
"	"	KOH	79.5	78	92	93
"	HCl	"			95	96
Factors.			1.78	1.55	1.695	0.99
Maltose. H <sub>2</sub> O.	H <sub>2</sub> SO <sub>4</sub>	NaOH	68	67	82	89
"	"	KOH	88	86	90.5	93
"	HCl	NaOH				102
"	"	KOH			94.5	98
Factors.			2.00	2.00	2.00	1.00

conditions. For the copper methods we used 0.02 per cent and for the Sumner method 0.05 per cent toluene-preserved solutions of the disaccharides. In order to calculate the degree of hydrolysis, we used factors which included (a) corrections for the glucose equivalents of the resulting mixtures and (b) estimations of the destruction of monosaccharides during the hydrolysis. The analyses by the three copper methods were made on the same hydrolysate. Comparisons were made against dextrose standards to which equivalent amounts of acid and alkali had been added. The reported values are averages of several determinations, and of these



the potassium hydroxide values are the more nearly correct for reasons already mentioned.

The Folin and Folin-Wu methods give slightly higher than theoretical values for the complete hydrolysis of sucrose, but the results by the other methods are satisfactory. The hydrolysis of lactose is not complete and the Folin-Wu and Folin methods give even lower values than the others. For the hydrolysis of this disaccharide the substitution of hydrochloric acid for sulfuric acid presents few advantages, but hydrochloric acid does increase the hydrolysis of maltose more perceptibly. It is worthy of note that the four methods give more uniform results with maltose hydrolysate than with those of the other disaccharides, probably because

TABLE VII.  
*Hydrolysis of Glycogen.*

Method.	Per cent hydrolyzed in:				
	$\frac{1}{2}$ hr.	$\frac{1}{2}$ hr.	1 $\frac{1}{2}$ hrs.	2 $\frac{1}{2}$ hrs.	5 hrs.
Folin-Wu.....	22.5	28.8	44.1	66.7	81.9
Folin.....			31.0		
Benedict.....	25.2	29.7	51.3	70.3	90.0
“ (HCl).....			69.4		
Sumner.....	27.6	40.5	62.2	78.2	85.2
“ (HCl).....			72.0		

the entire procedure has less effect on dextrose than on the other monosaccharides.

The differing values secured by the several copper methods on the same hydrolysates, despite corrections for all known deviations, indicate that there are formed during the hydrolysis sugars with glucose equivalents somewhat different from the ordinary hexoses. This fact must be borne in mind in attempting to interpret total sugar values secured on unknown materials such as blood and urine filtrates.

While heating disaccharides, in the concentrations mentioned, in 0.29 N acid solutions, for 1 $\frac{1}{2}$  hours on the water bath, results in the hydrolysis of over 90 per cent of the sugar, the results secured with a polysaccharide like glycogen under the same circumstances would be quite different. In the experiments recorded in Table VII, we determined the rate of hydrolysis of glycogen, using the

conditions of the total sugar method but varying the time of heating.

In this work 0.01 per cent toluene-preserved solutions of glycogen (C.P., Eastman) were used for the copper methods and 0.05 per cent solutions for the Sumner method. In all cases a factor of 1.11 was used to calculate the results, but since the latter represent only single analyses, not averages, they must be regarded as being only approximately true. We have made no corrections for possible loss of glucose during the longer periods of heating. A few experiments which we carried out indicate that such loss would be less than 5 per cent, for the 5 hour period, but exact values have not been determined.

It is evident that little more than one-half the glycogen is hydrolyzed in  $1\frac{1}{4}$  hours and about 85 per cent in 5 hours, longer heating periods being necessary for complete hydrolysis. Therefore, the use of hydrochloric acid presents certain advantages, as seen in Table VII. From the experiments so far reported, we see that it is impossible to specify a single concentration of acid or a definite period of hydrolysis which would satisfy every condition. The greater acid concentrations and longer periods of heating, necessary for the hydrolysis of the polysaccharides, might cause unnecessary destruction of sugar liberated from the hydrolysis of disaccharides and other easily hydrolyzed sugar compounds. Additional aid in specifying the conditions necessary for the total sugar determination should be forthcoming from a study of the results secured from blood and urine filtrates.

#### *Results with Blood Filtrate.*

In Table VIII we present a few analyses to show that the new method does correct the low values of the Folin-Berglund method. These analyses were made on Folin-Wu filtrates of whole blood. Any number of similar results could be given if necessary. We used 1 cc. of 2.6 N sulfuric acid, a similar quantity of silicate-free potassium hydroxide, and an hydrolysis period of  $1\frac{1}{4}$  hours. The addition of acid and alkali to the standard was discontinued because the use of potassium hydroxide abolishes the need of any correction for salt effects.

In Table IX we have assembled some data on the effects of variations in the length of the hydrolysis period. The Sumner

method used for this purpose was not that of Sumner and Graham (13). Their method is too cumbersome and absolutely unadapted to the total sugar procedure. We felt the need of some more applicable modification of Sumner's method and, after discussing the matter with Professor Sumner, we decided to use the following procedure for analysis of the Folin-Wu filtrate.

Place 5 cc. of the filtrate in a modified Folin-Wu blood sugar tube. (These tubes can be made by enlarging the bulbs of ordinary tubes to accommodate 8 cc. instead of 4 cc.) Add 3 cc.

TABLE VIII.  
*Blood Sugar (in Mg. Per Cent).*

Free.		Total by new method.		Total by Folin-Berglund method.
Folin-Wu.	Folin.	Folin-Wu.	Folin.	Folin-Wu.
82.3		93.3		
91.7		105.4		
90.8		102.5		
90.7		98.5		
101.5		115.6		
92.7		107.1		
87.0		121.2		66.2
98.5		118.7		61.8
100.5		122.0		59.5
90.3	82.9	114.2	120.4	
88.4	81.9	95.2	100.5	
	117.6		126.5	

of a reagent which is twice as concentrated as that recommended by Sumner for urine analysis. Then heat the tubes for 5 minutes on the boiling water bath, cool to room temperature, dilute to 25 cc., and mix. The standard sugar solutions to be used are 5 cc. of either 0.01 or 0.02 per cent dextrose.

In the preparation of the concentrated reagent, one may encounter a little difficulty in dissolving the dinitrosalicylic acid, but this trouble can be overcome with care. It is necessary to use 5 cc. of the blood filtrate because of the relatively low sensitivity of the dinitrosalicylate reagent. The color developed by 2 cc. of blood filtrate is too weak for accurate comparison. Our main

TABLE IX.  
*Sugar of Blood Filtrate (in Mg. Per Cent).*

Experiment No. and sample.	Method.	Free sugar.	Combined sugar determined by hydrolysing for:							
			$\frac{1}{2}$ hr.	$\frac{1}{2}$ hr.	$\frac{1}{2}$ hr.	1 $\frac{1}{2}$ hrs.	2 $\frac{1}{2}$ hrs.	5 hrs.	10 hrs.	
1. Normal.	Folin-Wu.	85.5	11.1		12.2	14.4	20.1			
	Benedict.	73.6	15.7		19.3	20.4	22.5			
2. Normal.	Folin-Wu.	87.7				10.6	15.9	19.7		
	Benedict.	81.7				12.8	14.1	25.6		
	“ (HCl).					18.3	10.9			
3. 3rd day of fast.	Folin-Wu.	71.8				27.8		23.8		
	Benedict.	69.7				35.8		21.6		
	Sumner.	78.4				35.2		35.2		
4. Fed 100 gm. glucose after Experiment 3.	Folin-Wu.	191.9				13.1		31.9		
	Benedict.	199.0				5.9		19.0		
5. a. b. a. b. a. b.	Folin-Wu.	95.7	6.6	9.8		14.5	17.6	19.3		
	“		8.3	13.3		15.8	17.5	23.8		
	Benedict.	93.2	17.8	21.3		29.8	33.8	31.3		
	“		16.8	27.8		30.8	33.3	29.3		
	Sumner.	97.2				16.1		21.8		
	“					18.1		25.3		
6. a. b. a. “ b. “ a. “ b. “	Folin-Wu.	90.8				17.7	16.6	12.0	20.3	
	“					32.4	42.1	45.1	58.9	
	Benedict.	86.5				12.3	11.9	3.0	9.1	
	“ (HCl).							8.5		
	Benedict.					19.8	33.6	32.5	39.1	
	“ (HCl).							40.4		
	Sumner.	89.7				21.4	26.0		26.0	
	“ (HCl).							14.9		
	Sumner.					45.4	57.4		71.6	
	“ (HCl).							53.9		

a = normal sample; b = same sample with added glycogen.

purpose in devising this method was not to displace the widely used copper methods, but merely to gain more information about the behavior of blood sugar. In an earlier paper (14), one of us

presented reasons for the choice of Sumner's method in preference to the older Benedict picrate method.

The use of the various blood sugar methods, in the determination of total sugar in blood filtrate, presented no difficulties except in the case of the Benedict copper method. With this method one is likely to encounter a turbidity from the formation of a difficultly soluble salt, after the addition of the arsenotungstic acid reagent. The trouble may be avoided by agitating the mixture immediately after the addition of the acid reagent, and allowing it to stand exactly 1 minute, before diluting and mixing. Undoubtedly other means of avoiding this turbidity may be found, but the described practice has proved quite satisfactory in our experiments. It takes more than 1 minute for enough of the precipitate to form to prevent its complete resolution upon diluting. Moreover, full color is developed in 1 minute because the reduced copper is not present in the form of a precipitate.

The apparent combined sugar values increase uniformly as the period of hydrolysis is lengthened to  $1\frac{1}{4}$  hours, but thereafter the results are less uniform. A few of the samples which have been heated for  $2\frac{1}{2}$  hours begin to show decreased values, although most samples show appreciable increases. After 5 or 10 hours hydrolysis a larger number of samples shows a loss of reducing substances but others still show large increases. In Experiment 6 (Table IX) we notice decreased values after 5 hours, followed by increased ones after 10 hours. Such results might be due to the destruction of free sugar, early in the hydrolysis, followed by the continued liberation of more free sugar from polysaccharides or other difficultly hydrolyzable substances.

Concerning the question of the possible presence of glycogen in blood filtrates, we call attention to Experiments 5 and 6. In the first of these experiments we added to an aliquot of the original blood an equal volume of 0.01 per cent, and in the second one, 0.05 per cent glycogen solution, in place of an equivalent amount of water during the laking of the blood. In the first case, excluding some of the Benedict values, we secured doubtful evidence of the presence of the added glycogen, by  $1\frac{1}{4}$  and 5 hours hydrolyses. However, the amount of glycogen added was too small to give conclusive data, for, even if the entire amount were hydrolyzed, only 11 mg. per cent of free sugar would result. This increase would make a

change of only 1.5 to 2.0 mm. in the colorimeter reading for the total sugar. On the other hand, the results of the second experiment leave no room for doubt. In this case every analysis but one shows over 50 per cent of the expected quantity of added glycogen to be present. Therefore, it is possible for a part of the added glycogen to escape precipitation with the protein, and the contrary results of Folin and Berglund may be attributed to their incorrect method of hydrolysis. The amount of glycogen in blood filtrates is probably less than that in the original blood, because the increases in total sugar, noted in our two experiments, are less than the theoretical values calculated from the data of Table VII. These facts are important in the interpretation of total sugar values.

The Sumner method gives the highest and the Benedict method the lowest values for glycogen recovery, but these relations do not always hold for the natural combined sugar of blood filtrates. It is to be remembered that we are not dealing with glycogen alone, but with a mixture, and that we are not only hydrolyzing combined sugar, but also probably destroying reducing substances. Hence it is very difficult to choose a definite period of hydrolysis for the total sugar method. For the present we feel that it is best to continue the  $1\frac{1}{4}$  hours period of Folin and Berglund. Analyses would be of greater value if they also included a longer period of hydrolysis, say 5 to 10 hours.

The possible occurrence of glycogen in blood filtrates might be further tested by the behavior of blood sugar upon treatment with an alkali. For this purpose 8 cc. of blood filtrate were heated in the customary total sugar tube on the water bath for 2 hours with 1 cc. of 2.6 N potassium hydroxide solution. The tube was then cooled and its contents neutralized with 1 cc. of equivalent sulfuric acid solution. Free and total sugar analyses were conducted on this mixture by the usual procedures. We believe that these experiments did not give sufficiently accurate data to warrant publication. The amounts of free and combined sugars which remain after the treatment with alkali, are too small to be determined with accuracy by any of the usual methods. As a result we are in doubt about the complete destruction of glucose under these circumstances. Moreover, experiments on pure glycogen indicated that this polysaccharide is not totally unaffected by the process

when such dilute solutions are employed. The further investigation of the nature of combined sugar in blood filtrate remains for the future.

*Results with Filtrates of Normal Urine.*

Table X contains a few results of the application of the new total sugar method to urine filtrates. The procedure in these cases is entirely similar to that used for the blood filtrates, except that some form of decolorization is often necessary before the final colorimetric determinations can be made, especially for the copper methods. With pure glucose solutions both bone-black and Lloyd's reagent seem to be satisfactory provided small enough

TABLE X.  
*Urine Sugar (in Mg. per Hour).*

Free.			Total by new method.		
Folin-Wu.	Folin.	Sumner.	Folin-Wu.	Folin.	Sumner.
18.8	15.5	10.6	29.6	24.3	19.4
14.1	11.4	10.4	16.8	15.5	12.3
126.8	119.8	121.5	297.5	322.6	247.7
22.7	12.6	18.4	142.3	177.7	110.4
11.9	7.2	12.4	43.9	53.3	32.9
16.0		20.0	35.2		40.2
27.1		36.3	39.6		41.6
31.8		26.7	33.6		36.0

amounts be used (*cf.* also Kolthoff (15)). Hence one may select the same substance used in the preparation of the original filtrate, or bone-black may be used for all methods, with little difference in results. The Benedict analyses were performed on the Folin-Berglund filtrates.

The third, fourth, and fifth sets of analyses were made on samples of urine from a young man who had eaten 100 gm. of sucrose. We have not expanded the table to include Benedict values, because we feel that added data are unnecessary. Our results for total sugar on hundreds of urine samples, with the four methods, have always been satisfactory. In fact, the only samples of blood or urine which have ever given lower total than free sugar values

have been samples secured under very abnormal experimental conditions.

If we may use the relatively small number of analyses of Table XI as a criterion, it is evident that a 2½ hours hydrolysis period is most suitable for urine analysis. The Sumner values for combined

TABLE XI.  
*Sugar of Urine Filtrates (in Mg. per Hour).*

Experiment No. and sample.	Method.	Free sugar.	Combined sugar determined by hydrolyzing for:					
			½ hr.	¾ hr.	1½ hrs.	2½ hrs.	5 hrs.	10 hrs.
1. Normal.	Folin-Wu.	36.3			17.2		21.4	
	Benedict.	39.4			17.0		22.4	
	Sumner.	41.7			4.2		5.1	
2. 3rd day of fast.	Folin-Wu.	12.8			4.7		9.3	
	Benedict.	16.2			5.8		7.2	
	Sumner.	12.5			2.4		3.5	
3. After a night's fast.	Folin-Wu.	22.7	5.7	7.5	10.7	14.9	4.0	
	Benedict.	22.3	5.3	7.8	9.2	12.0	12.9	
	Sumner.	22.7	1.2	1.7	1.7	1.7	2.8	
4. Normal.	Folin-Wu.	17.1			9.9	15.1	13.1	13.1
	Benedict.	19.1			8.6	9.1	12.6	13.1
	Sumner.	21.9			3.0	6.6	4.1	3.3
5. Normal.	Benedict.	21.3			9.3	27.4		
	“ (HCl).				11.3	35.7		
	Sumner.	24.4			4.1	4.9		
6. Normal.	“ (HCl).				4.1	4.7		
	Folin-Wu.	20.2			12.4			
	Benedict.	17.9			11.5			
	“ (HCl).				13.6			
	Sumner.	20.8			7.5			
	“ (HCl).				8.1			

sugar are invariably lower than similar values secured by the copper methods.

The choice of the best colorimetric method for determining “sugar” of normal urine must be left for the future because at present little is known about the nature of the substances being



determined. Since the several methods give varying results on different samples of urine, it is best, for the present, to use as many of these methods as possible. The experiments recorded in Table XII show that 20 to 30 per cent of the free reducing substance of urine is not destroyed by heating 2 hours on the water bath with 0.29 N alkali. Most of the combined sugar seems to be unaffected by the same process. As explained previously, accurate data of a similar nature for blood filtrate are not available. We are certain, however, that the "free sugar" of urine is more resistant to alkali treatment than the free sugar of blood. (Cf. also the experi-

TABLE XII.  
*"Urine Sugar" and Alkali Treatment.*

Experiment No. and sample.	Mg. sugar per hr.		
	Folin Wu.	Benedict.	Sumner.
1. Free.....	17.1	19.1	21.9
" after KOH.....	5.5	4.8	6.8
Combined.....	9.9	8.6	3.0
" after KOH.....	7.2	6.4	4.3
2. Free.....	16.4		19.5
" after KOH.....	4.6		3.3
Combined.....	15.7		7.7
" after KOH.....	8.4		6.7
3. Free.....	33.6	34.3	
" after KOH.....	7.4	8.0	
Combined.....	18.9	15.0	
" after KOH.....	12.6	10.9	

ments of Sumner (16) on original urine.) In the near future, we hope to be able to present other facts concerning the nature of sugar of normal urine.

#### SUMMARY.

A method is described for the determination of total sugar in blood and urine filtrates. The experimental data, which serve as a basis for the method, are given. The data include glucose equivalents for known sugars and estimations of the destruction of sugar by acid and alkali.

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## GLYCOLYSIS IN LEUCEMIC BLOOD.\*

By HENRY LENZEN SCHMITZ AND EUGENE CHELLIS GLOVER.

(From the Medical Service of the Collis P. Huntington Memorial Hospital of Harvard University, Boston.)

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In a recent contribution from this hospital Falcon-Lesses (1) reported the results of his investigation of the disappearance of glucose *in vitro* at 37°C. in the blood from six patients with chronic myelogenous leukemia and one with chronic lymphatic leukemia. He found that glycolysis in the blood of the former patients was two or three times as rapid as normal, so that the process was often complete in 2 hours. The most rapid rates occurred in the cases with the highest white blood cell counts, but there was also some evidence to suggest that very immature or atypical white cells hastened the process more than mature or typical ones. The slowest rate for any of the seven cases took place in the blood from the patient with chronic lymphatic leukemia, who also had the lowest white blood cell count. It was a question, therefore, whether this slower rate could be dependent upon the low white cell count or whether the lymphocytes influenced the rate in a different manner from the bone marrow white cells.

We have given further consideration to this problem and have studied the influence of the number, the type, and the immaturity of the white blood cells upon the glycolytic rate in leucemic blood. In addition the investigation has been extended to include a study of the effect of respiration upon the glycolytic process in both normal and leucemic whole blood. Data are presented for fourteen cases, seven of chronic lymphatic leukemia and seven of chronic myelogenous leukemia. For purposes of

\* The expenses of this investigation have been defrayed in part by a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases. A part of the work was done under a Bullard Fellowship (George Cheyne Shattuck Memorial Fellowship).

comparison the rates in the bloods of ten normal individuals are given. Falcon-Lesses presented some evidence suggesting that the rate of glycolysis was more rapid the higher the initial concentration of glucose. It was thought best to study this phase of the subject somewhat more intensively in order to determine whether the initial blood sugar level must be taken into account when comparing the rates of glycolysis of different samples of blood.

### *Methods.*

None of the experiments was performed with strict asepsis, not only because the duration was so short as to preclude any significant bacterial growth, but also because Falcon-Lesses showed that aseptic conditions did not alter the results. Blood was drawn from an arm vein and discharged into one or more Erlenmeyer flasks of 50 cc. capacity. 5 to 8 cc. of blood were placed in each flask. Heparin was used to prevent clotting and it was found that this was most conveniently employed in the form of a solution of which 0.05 cc. was equivalent to 1.0 mg. of the dry powder. Of this solution 0.05 cc. was sufficient to prevent the coagulation of 5 cc. of normal blood. Twice this amount was used for leucemic blood because leucemic blood *in vitro* tends to clot more rapidly than normal. Falcon-Lesses has shown that such an amount of heparin does not affect the rate of glycolysis. To the blood in the Erlenmeyer flask, therefore, the proper amount of heparin solution was added, a 1 cc. sample was withdrawn, and a protein-free filtrate immediately prepared from it by the Folin-Wu method (2). The flask, containing the remainder of the blood, was stoppered with cotton to prevent loss by evaporation and placed in the incubator at 37°C. Thereafter protein-free filtrates were prepared from 1 cc. of blood taken from the incubator specimen at half-hour or hour intervals. Sugar determinations were made upon the protein-free filtrates, thus obtained, by Folin's new method (3, 4). It should be remembered that this method usually gives lower values than does that of Folin and Wu.

### *Glycolysis in Normal Blood.*

In Table I are presented the results of seventeen experiments upon the blood from ten healthy individuals. The rates of

glycolysis obtained varied between 15 and 23 mg. per 100 cc. of blood per hour. An average normal rate of glycolysis has not been calculated from these results for it was considered more important to establish and to bear in mind the upper and lower limits of normal.

After 4 or 5 hours the reducing substances in the blood had usually fallen to a level somewhere between 10 and 5 mg. per 100 cc. of blood. This level marks, for Folin's new method, the

TABLE I.  
*Rate of Glycolysis in Normal Blood at 37°C.*

Case No.	Mg. of glucose per 100 cc. blood.						Rate of glycolysis in mg. glucose per 100 cc. blood.				
	Initial value.	Values after:					1st hr.	2d hr.	3d hr.	4th hr.	Average rate per hr.
		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.					
1a	70	54	38	27	10	9	16	16	11	17	15
1b	77	59	45	29	16	11	18	14	16	13	15
1c	76	57	42	23	12	10	19	15	19	11	16
1d	89	70	53	33	16	9	19	17	20	17	18
1e	125			65							20
2	139	125	105				14	20			17
3a	83			29							18
3b	112			57							18
4	80	61		13			19				22
5	98	72	53	31			26	19	22		22
6		44	31	14	5	5		13	17		15
7	96			45							17
8a	129			68							20
8b	80		37								22
9a	73		39								17
9b	88			19							23
10		60	37	14	8			23	23		23

end of the process of glycolysis. The remaining 10 to 5 mg. represent non-glucose substances which reduce the copper reagents (4). Even prolonged standing will not reduce this value further.

#### *Effect of Initial Concentration of Glucose upon Rate of Glycolysis.*

As has been pointed out by numerous investigators, the disappearance of glucose proceeds at a fairly uniform rate. In the first experiment recorded in Table I, for example, the rates for

TABLE II.

*Effect of Initial Concentration of Glucose upon Rate of Glycolysis.*

Case No.	Mg. glucose per 100 cc. blood.						
	Initial value.	Values after:					
		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	Average rate of glycolysis per hr.
1. Normal individual.							
a. Fasting.....	70	54	38	27	10	9	15
b. 45 min. after ingestion of 200 cc. Karo corn syrup.....	100	83	63	47	33	19	16
2. Normal individual.							
a. At normal glucose level.....	89	70	53	32	16		18
b. Same plus added glucose.....	265	241	222	206	183	160	21
3. Normal individual.							
a. At normal glucose level.....	80	61		13			22
b. Same plus added glucose.....	182	153		100			27
4. Normal individual.							
a. At normal glucose level.....	112			57			18
b. Plus 60 mg. ".....	184			123		70	19
c. " 160 " ".....	262			208		160	18
5. Normal individual.							
a. At normal glucose level.....	80		37				22
b. Plus 60 mg. ".....	143		99		61	19	21
c. " 160 " ".....	222		186		149	106	19
6. Aleucocythemic myelogenous leucemia.							
a. At normal glucose level.....	102	86		49			18
b. Plus 50 mg. ".....	167	148		113			18
c. " 100 " ".....	234	215		186			16
7. Myelogenous leucemia.							
a. At normal glucose level.....	57	19					38
b. Plus 60 mg. ".....	109	73	36				37
8. Myelogenous leucemia.							
a. Plus 100 mg. glucose.....	172	88					84
b. " 200 " ".....	250	175					75

the first 4 hours were 16, 16, 11, and 17 mg. per 100 cc. of blood respectively. This has an important bearing upon the question of the influence of glucose concentration upon the rate of glycolysis. If the glycolysis proceeds at such a uniform rate, then the sugar concentration, which is steadily decreasing, can have little if any effect upon it, at least within the limits studied. Furthermore, although the initial blood sugar levels in these experiments ranged between 70 and 139 mg. per 100 cc. of blood, there is no evidence of any correlation between the height of the initial sugar concentration and the rate of glycolysis.

TABLE III.

*Rate of Glycolysis in Myelogenous Leucemia. (Whole Blood at 37°C.)*

Case No.	R.B.C. count in millions.	W.B.C. count in thousands.	Mg. glucose per 100 cc. blood.					
			Initial value.	Values after:				Average rate of glycolysis per hr.
				$\frac{1}{2}$ hr.	1 hr.	1 $\frac{1}{2}$ hrs.	2 hrs.	
1a	3.3	395	92	64	21	11	10	71
2	2.6	186	172	126	88			84
3	1.9	180	92	74	33			59
4a	3.8	161	127				29	49
4b	3.6	160	71	53	38	24	7	32
5	3.7	100	81		51		12	35
6	3.9	54	57		19			38
1b*	2.5	13	78		51		26	26
7	3.1	3	103		86		66	18

\* The reduction of this patient's white blood cell count was due to Roentgen ray treatment.

The influence of the initial sugar level upon the glycolytic rate was studied more specifically in two ways. First was tested the rate of glycolysis of the blood drawn from a normal individual in the fasting state compared with the rate of glycolysis of the blood drawn from the same individual 45 minutes after the ingestion of 200 cc. of Karo corn syrup. Secondly, in seven experiments the glycolytic rate was obtained after the addition of glucose to a sample of blood and was compared with the glycolytic rate of the same blood at its normal sugar level. Of these latter experiments, one was performed upon the blood from a case of aleucocythemic myelogenous leucemia and two upon blood



from cases of myelogenous leucemia with white blood cell counts above 50,000 per c. mm. The results are presented in Table II.

In but one instance (Case 3) was there any marked difference between the rate of glycolysis of the same blood at a high and a low initial sugar concentration. It is especially significant that there was no tendency for the rate to change in any one direction. Slightly faster rates at the higher sugar levels occurred no more frequently than did slightly slower ones. In most cases the rates were practically the same. The results of these experiments and the uniformity of the rate of glycolysis from hour to hour seem to confirm the conclusion of Macleod (5, 6), Kawashima (7), and others, that sugar concentration has but little effect upon the rate of glycolysis.

#### *Glycolysis in Myelogenous Leucemia.*

Table III gives the results of nine experiments upon the blood from seven cases of myelogenous leucemia. These experiments confirm the observations of Bürger (8) and Falcon-Lesses (1) that glycolysis in the blood from cases of chronic myelogenous leucemia with high white blood cell counts is more rapid than normal. The rates obtained in the first experiment upon Case 1 and for Case 2 are more rapid than any observed by Falcon-Lesses.

Furthermore, it is evident from the data in Table III that the number of white blood cells and the rate of glycolysis tend to run parallel.

These cases of myelogenous leucemia were grouped by Dr. George R. Minot according to the total number and character of the immature cells in the venous blood so that we might determine whether or not there was any relationship between the immaturity of the white blood cells and the rate of glycolysis. For this purpose Dr. Minot arranged the three groups described in connection with Table IV. A fourth group was also established for cases in which almost all the cells are myeloblasts and many of them pathological, but there is no case in the series which fulfils its requirements.

It seems evident from the results presented in Table IV that the rate of glycolysis tends to be greater, the more immature the white blood cells. It is also apparent that one cannot hope to

predict, except in a most general way, what the rate of glycolysis in a given sample of blood will be from the number and degree of immaturity of the white blood cells. Other variable factors, such as the number and character of the red blood cells, also affect the rate of glycolysis. The red blood cells can be shown to exert an influence upon the rate in a suspension of white blood cells. This will be described in a subsequent paper.

TABLE IV.

*Rate of Glycolysis in Myelogenous Leucemia. (Whole Blood at 37°C.)*

Case No.	R.B.C. count in millions.	W.B.C. count in thousands.	Immaturity.*	Average rate of glycolysis per hr. in mg. glucose per 100 cc. blood.
2	2.6	186	+++	84
1a	3.3	395	+++	71
3	1.9	180	+++	59
7	3.1	3	+++	18
4a	3.8	161	++	49
6	3.9	54	++	38
5	3.7	100	++	35
4b	3.6	160	+	32
1b	2.5	13	+	26

\* The symbols in the column headed "immaturity" have the following significance. + Indicates that the immature cells are present in relatively low percentage and that they are chiefly myelocytes of a type that approaches the polymorphonuclear (Myelocyte C of Sabin *et al.* (11)). There are practically no myeloblasts or atypical cells in this group. ++ In this group the percentage of immature cells is greater (often 50 per cent) and one often finds more immature myelocytes than in the + group. There are a few myeloblasts but atypical cells are rare. +++ The immature cells are not necessarily present in greater percentage than in the ++ group but there are distinctly more very young myelocytes (Myelocyte A of Sabin *et al.*). Myeloblasts are more numerous than in the ++ group (often 8 per cent) and rarely a grossly pathological cell is seen.

#### *Glycolysis in Lymphatic Leucemia.*

The results of testing the glycolysis of the blood from the seven cases of chronic lymphatic leucemia are presented in Table V. The most striking thing shown is that the rate of glycolysis in six out of the seven cases was below the upper limit of normal and in the seventh case was only slightly more rapid than normal;

and yet the white blood cell count in five of the cases was 100,000 per c.mm. or more.

How can this marked difference in behavior between the blood of lymphatic leukemia and that of myelogenous leukemia be accounted for? Two possibilities suggest themselves. Either the lymphoid cells have a slower rate of glycolysis than the myeloid cells, or the former have a more rapid respiration and reconvert a larger amount of lactic acid to glucose (9). The experiments dealing with the effect of KCN upon glycolysis, presented in

TABLE V.  
*Rate of Glycolysis in Lymphatic Leucemia. (Whole Blood at 37°C.)*

Case No.	R.B.C. count in millions.	W.B.C. count in thousands.	Immaturity.*	Mg. glucose per 100 cc. blood.						
				Initial value.	Values after:					Average rate of glycolysis per hr.
					½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	
1	1.7	109	+++	120	106	90				30
2	3.9	16	++	88		63	54	33		18
3	4.9	90	++	81		65	44	24	10	18
4	0.9	293	+	108		88	72	50		19
5a	3.0	196	+	101		86	62	44	30	18
5b	2.4	108	+	116		107	100	95	85	8
6	1.2	203	+	102		87	71	56		15
7	3.7	21	+	99		71			14	

\* The symbols in this column have the following significance: + A small number of the lymphocytes are somewhat less mature than normal cells. ++ Many are somewhat less mature than normal cells. +++ Most of the cells are very immature. All of the bloods contained not less than 90 per cent of lymphocytes.

Table VI and discussed later on, indicate that this latter possibility is not probable.

In these seven cases, there is obviously no correlation between the number of white blood cells and the rate of glycolysis.

In the second experiment upon Case 5 (Table V) the glycolytic rate was markedly slower than normal. This fact may be dependent upon the very low red blood cell count. Kawashima (7), for example, has shown that in normal rabbit blood the degree of glycolysis and the number of red blood corpuscles run parallel. In Case 5b (Table V), at the time of the test, there was only

one-half the normal number of red blood cells, and though the white blood cell count was 100,000 per c.mm., with 96 per cent

TABLE VI.

*Effect of 0.001 N KCN upon Rate of Glycolysis in Normal and Leucemic Blood.\* (Whole Blood at 37°C.)*

Case No.	Diagnosis.	Immaturity of white blood cells.†	Average rate of glycolysis per hr. without KCN.‡	Rate of glycolysis with 0.001 N KCN.							Rate with KCN divided by rate without KCN.	
				Initial value.	Values after:							Average rate per hr. in mg. glucose per 100 cc. blood.
					½ hr.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	4 hrs.		
1	Normal.	0	22	91	66		44	27		21	0.95	
2	"	0	15	45	24		11	5	5	17	1.1	
3	"	0	17	86				33		18	1.1	
4	"	0	20	124				64		20	1.0	
5a	"	0	17	73			29			22	1.3	
5b	"	0	23	88				9		26	1.1	
6	"	0	20	125				60		22	1.1	
7	Myelogenous leucemia.	+++	84	167	90	24				143	1.7	
8	" "	+++	71	94	36	10	9			116	1.6	
9	" "	+++	60	89	47	6				83	1.4	
10	" "	++	35	60	12		9			48	1.4	
11	" "	+	32	78	43	10	9	6		68	2.1	
12	Lymphatic "	+++	30	108	91	74				34	1.1	
13	" "	++	18	81	60		37	19	8	21	1.2	
14	" "	+	15	71	46		21	8		25	1.7	
15	" "	+	8	96	79		59	51	43	13	1.6	
16	Aleucocythemic myelogenous leucemia.	+	26	76	43		20			28	1.1	
17	Aleucocythemic lymphatic leucemia.	+++	18	100	78		55	40		20	1.1	

\* In each instance the rate with KCN and the rate without KCN were determined simultaneously on different samples of the same specimen of blood.

† See foot-notes to Tables IV and V for the significance of these symbols.

‡ The readings from which these rates were obtained are presented in Tables I, III, and V.

lymphocytes, this increased number of lymphocytes evidently was not sufficient to make up for the reduction in rate resulting presumably from the anemia.

All the bloods were grouped by Dr. Minot in similar fashion to those of chronic myelogenous leucemia. In Case 1 the rate was slightly above normal even though the red blood count was below 2,000,000 per c.mm. and the white blood cell count no higher than in Case 5b, described above. There was, however, a very marked difference in the degree of immaturity of the lymphocytes in the blood of these two patients. Case 1 presented by far the greatest number of unusually immature lymphocytes of all seven. The cells indeed were so young and undifferentiated as to make it difficult to determine whether they were of lymphoid or myeloid origin. The diagnosis of lymphatic leucemia could be made with finality only from the multiple enlarged lymph nodes and from the examination of blood smears taken some weeks previously. Here, then, there seems to be evidence that very immature and atypical lymphoid cells hasten the glycolytic process more than mature or typical ones do. However, as in myelogenous leucemia, there are many variable factors that tend to obscure considerably the influence of the age of the cells. In order to obtain more significant data concerning this phase of the subject it is necessary to eliminate the variable factors as far as possible. This is being undertaken by studying the white blood cells alone instead of the whole blood.

#### *Effect of Potassium Cyanide upon Glycolysis.*

Two ways of determining the effect of respiration upon the glycolytic process suggested themselves. The first was to remove the supply of oxygen. This method because of the inherent power of the red blood cells to hold oxygen in the form of oxyhemoglobin was impracticable for use with whole blood. The second method was to arrest the respiration by chemical means. Warburg (10) has shown that hydrocyanic acid in concentrations from 0.00001 N to 0.001 N will retard all those processes in which the transfer of oxygen plays a part. A concentration of 0.001 N potassium cyanide, according to Negelein, will completely arrest respiration but does not appear to injure the cells (9).

In order to make sure that KCN would arrest the oxygen consumption of leucemic blood, which is much greater than that of normal blood, the following test was made. 12 cc. of blood, from

a patient with myelogenous leucemia, were divided into three portions. One portion was placed in the cup of a Van Slyke blood gas apparatus under a layer of caprylic alcohol, while the other two were placed in small Erlenmeyer flasks under paraffin oil and incubated at 37°C., after sufficient KCN had been added to one of them to make a concentration of 0.001 N. The oxygen content at the beginning of the test was found to be 14.4 volumes per cent. After 6 hours incubation, the oxygen content of the sample without KCN had fallen to 5.3 volumes per cent, whereas that of the sample containing KCN was not significantly changed. Thus the KCN completely arrested the consumption of oxygen by the blood cells. It was interesting to observe that the blood containing KCN remained bright red in color, whereas that without KCN turned dark as its oxygen was used up. Two other experiments, one of them with blood from a patient with lymphatic leucemia, yielded similar results.

This substance was therefore employed to produce anaerobic conditions, and a stock solution of 0.02 N was prepared. From this solution of KCN there was added to the sample of blood a sufficient amount to make the resulting concentration 0.001 N. A specimen of the same blood without KCN was tested simultaneously for comparison. Table VI gives the results of such experiments upon the blood of six healthy individuals, five typical cases of myelogenous leucemia, four cases of typical lymphatic leucemia, and one case each of aleucocythemic myelogenous leucemia and aleucocythemic lymphatic leucemia.

In normal blood the addition of 0.001 N KCN had very little effect upon glycolysis. In five of the six cases the rate of glycolysis with KCN was practically the same as the rate without it. In the five typical cases of myelogenous leucemia there was a very marked increase in rate when KCN was added to the blood, the rate with KCN varying from 1.4 to 2.1 times the rate without KCN. The blood from the four cases of typical lymphatic leucemia showed a much less definite increase in rate upon the addition of KCN than did that of myelogenous leucemia. In only two of the four cases was the increase of the same order of magnitude as in all of the cases of myelogenous leucemia.

If one considers that the difference between the rate of glycolysis with KCN and that without KCN represents the amount

of lactic acid reconverted into glucose as a result of the cell respiration, then one may look upon this difference as a measure of the magnitude of the respiration. Such reasoning would indicate that the white blood cells in the blood of the cases of myelogenous leucemia had a comparatively rapid respiration. Of the four cases of lymphatic leucemia, the cells in the bloods of two had a relatively rapid respiration while those of the other two respired very slowly. It is of interest that the lymphocytes in the two cases with slow respiration were the more immature. There is thus no evidence that the lymphocytes have a greater respiration than the bone marrow white cells. Indeed, our experiments would seem to indicate that they have, if anything, a smaller respiration. It seems likely, therefore, that any difference that exists between the rate of glycolysis in the blood in lymphatic leucemia and that in myelogenous leucemia is due to the greater glycolytic strength of the myeloid cells rather than to a greater respiratory activity of the lymphocytes associated with more rapid reversion of lactic acid into glucose. In the two cases of aleucocythemic leucemia there was only a slight difference, if any, between the rates obtained with and without KCN.

#### SUMMARY.

1. The rate of glycolysis in normal blood, as determined from the study of ten normal individuals, varies approximately between 15 and 23 mg. per 100 cc. of blood per hour. The initial concentration of glucose, within a range of from 60 to 250 mg. per 100 cc., does not affect the rate of glycolysis in normal or leucemic blood.

2. In chronic myelogenous leucemia, the rate of blood glycolysis is more rapid than normal, except in an aleucocythemic stage. It may be as rapid as 84 mg. per 100 cc. per hour. The number of white blood cells and the rate of glycolysis tend to run parallel. The degree of immaturity of the white blood cells and the rate of glycolysis also tend to run parallel.

3. In chronic lymphatic leucemia, the rate of blood glycolysis is seldom more rapid than normal. But when the lymphocytes are very immature, the rate may be slightly rapid.

4. Potassium cyanide, in a concentration of 0.001 N, causes a marked increase in the rate of blood glycolysis in myelogenous

leucemia; in one case the rate was more than doubled. It causes a less definite increase in the rate of glycolysis in lymphatic leucemia blood, and has very little effect upon the rate in normal blood.

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# ON THE NITROGEN METABOLISM IN EXPERIMENTAL SUBACUTE ARSENIC AND ANTIMONY POISONING.

By EMIL PRIBYL.\*

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

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The variations of nitrogen metabolism, especially urea, in diseases of the liver due to bacterial and chemical causes as well as conditions produced by removing the liver or cutting it off from circulation partially or completely have been studied of late with varying results. The effect of fatty degeneration of the liver lobules produced chemically by P, As, Sb, alcohols,  $\text{CHCl}_3$ , ether, cyanohydrogen, hydrazine, trinitrotoluene, dinitrobenzene, on the non-protein nitrogen of blood and urine has been investigated by various workers. It is without doubt, that many other chemicals may produce degeneration in liver cells. Wells (1) pointed out that any poison which does not directly cause death, but which causes a severe injury to the liver cells without at the same time destroying the autolytic enzymes, so that the cells die and undergo rapid autolysis, may produce a condition identical with or similar to acute yellow atrophy.

In 1876 Kossel (2) found an increase of total non-protein nitrogen in urine in liver degenerations following experimental arsenic poisoning and suggested it to be due to urea increase. Löffler (3) working with isolated liver found no abnormal variation in urea formation in phosphorus poisoning and consequent fatty degeneration of liver lobules; in chloroform and alcohol poisoning, he found that the urea formation is actually affected. Also Luciano (4) reported a decrease of urea formation in chloroform poisoning. Marshall and Rowntree (5) noted definite and sometimes marked increase in non-protein nitrogen, urea, and amino acid nitrogen in

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\* Fellow of the International Education Board.

I wish to express my gratitude to Professor Folin for his permission to carry on this work in his laboratory, and for his advice and interest, and to the International Education Board for making this opportunity possible.

the blood in phosphorus poisoning. Jackson and Pearce (6) working with dogs with necrosis of liver produced by hemotoxic serum did not notice any variations in urea formation and concluded that this "factor of safety" is very important in the chemistry of liver diseases. The ratio of urea, ammonia, and amino acid is not always disturbed.

The results are far from uniform and with a view to elucidate the relation of urea to non-protein nitrogen in blood and urine in degeneration of liver, the following experiments were undertaken.

Throughout rabbits were used as experimental animals and in one group a subacute poisoning by sodium arsenite and in the other by antimony potassium tartrate was produced. During the period of experiments the rabbits were fed on 75 cc. of milk and 50 gm. of glucose per kilo of weight of rabbit per day in two meals, one at 10 a.m. and one at 10 p.m. When the animals were unable to take food they were fed by stomach tube. The urines were collected from metabolism cages every 24 hours and preserved with toluene. At regular intervals blood was taken from the marginal ear vein. The dose of sodium arsenite and antimony potassium tartrate given *per os* in solution was 10 and 15 mg., respectively, per kilo of body weight. Rabbit 1 was given the toxic dose of 10 mg. per kilo of body weight all at once and it died 3 days later; to prolong the period of poisoning both of the poisons were, therefore, administered in several increasing doses in the other rabbits.

*Chemical Methods Used.*—The preparation of protein-free blood filtrate has been made by the Folin-Wu method (7). Non-protein nitrogen and urea nitrogen were determined by Folin's colorimetric methods (7); also, for the determination of total nitrogen, urea, and ammonia nitrogen, Folin's colorimetric methods were used (8-10).

Tables I and II show the average figures for blood and urine before and after the poisoning. The figures under "before" represent the average data calculated for a period of 6 days prior to the administration of poison.

The non-protein nitrogen in the blood of normal rabbits varied between 30.74 and 33.82 mg., the urea nitrogen between 12.28 and 13.21 mg. per 100 cc. of blood. The urea nitrogen quotient 
$$\frac{\text{urea nitrogen}}{\text{non-protein nitrogen}}$$
 in the blood of normal rabbits is between

37.4 and 43.3 per cent. The daily output of total nitrogen in urine in the period prior to administration of poison was between 269 and 675 mg., the urea nitrogen quotient between 72.1 and 85.3 per cent. The ammonia nitrogen excreted in a 24 hour period was between 2.75 and 7.66 mg., the ammonia nitrogen quotient was 0.4 to 1.9 per cent.

*Non-Protein Nitrogen, Urea, and Ammonia Nitrogen in Blood and Urine after Administration of Sodium Arsenite.*—There was

TABLE I.

*Showing the Average Data for Each Rabbit before and after Administration of Sodium Arsenite.*

Rabbit No.	Duration of poisoning.	Loss of body weight.		Whole blood per 100 cc.			Urine of 24 hrs.				
				Non-protein N.	Urea N.	Urea N quotient.	Total N.	Urea N.	Urea N quotient.	NH <sub>3</sub> -N.	NH <sub>3</sub> -N quotient.
				mg.	mg.	per cent	mg.	mg.	per cent	mg.	per cent
1	4	15	Before.	33.82	12.93	39.1	269	207	76.9	5.17	1.9
			After.	36.50	18.08	50.2	370	297	80.4	5.83	1.5
2	12	10	Before.	30.74	13.33	43.3	622	480	77.1	2.94	0.4
			After.	45.46	21.24	47.2	650	464	71.4	3.42	0.5
3	8	22	Before.	32.81	13.19	40.2	341	291	85.3	4.16	1.2
			After.	42.78	25.84	61.5	651	546	83.9	2.59	0.4
4	16	35	Before.	31.01	13.21	42.6	335	264	78.8	6.28	1.8
			After.	36.24	15.30	42.5	509	417	81.9	7.85	1.5

a marked increase of non-protein nitrogen in the blood in all four rabbits (Table I). The urea nitrogen also showed a rise in all cases. The urea nitrogen quotient increased in all cases except in Rabbit 4 where it was of almost the same value as before the poisoning. The rise of non-protein nitrogen in the blood of poisoned rabbits (except No. 4) was due to the rise of urea nitrogen. The changed blood picture was not associated with the same changes in urine. The total nitrogen in all cases increased, also the urea nitrogen with the exception of Rabbit 2. The urea

nitrogen quotient increased in Rabbits 1 and 4, and decreased in the other two cases. The ammonia nitrogen increased but not proportionally to total nitrogen; the ammonia nitrogen quotient was, therefore, lower.

*Non-Protein Nitrogen, Urea, and Ammonia Nitrogen in Blood and Urine in Rabbits after Administration of Antimony Potassium Tartrate.*—In general the rise with antimony potassium tartrate was not so great as with sodium arsenite, but still there was a

TABLE II.

*Showing the Average Data for Each Rabbit before and after Administration of Antimony Potassium Tartrate.*

Rabbit No.	Duration of poisoning.	Loss of body weight.		Whole blood per 100 cc.			Urine of 24 hrs.				
				Non-protein N.	Urea N.	Urea N quotient.	Total N.	Urea N.	Urea N quotient.	NH <sub>3</sub> N.	NH <sub>3</sub> N quotient.
	days	gm.		mg.	mg.	per cent	mg.	mg.	per cent	mg.	per cent
I	10	105	Before.	31.87	12.46	39.0	561	466	83.0	2.75	0.4
			After.	34.69	13.99	40.3	664	485	71.5	5.01	0.7
II	13	20	Before.	32.80	12.28	37.4	495	387	78.1	7.21	1.4
			After.	33.78	13.50	39.9	542	424	78.2	6.34	1.1
III	7	15	Before.	32.00	12.46	38.9	675	557	82.5	7.66	1.1
			After.	38.70	15.66	40.4	479	394	82.2	6.52	1.3
IV	22	140	Before.	30.72	12.98	42.2	280	202	72.1	4.47	1.5
			After.	36.44	14.30	39.24	527	428	81.2	5.62	1.0

rise in non-protein nitrogen and urea nitrogen in blood and urine (Table II). The rise of non-protein nitrogen was not entirely on account of the rise of urea nitrogen as was the case in arsenic poisoning. The difference in urea nitrogen quotients was only 1 to 2 per cent, while the difference in quotients in arsenic experiments was 4 to 21 per cent.

The total nitrogen in urine increased in all cases except in Rabbit III. The urea nitrogen quotient was almost the same in urines of Rabbits II and III; in Rabbit I there was a fall and in

Rabbit IV a marked decrease. The ammonia nitrogen quotient fell in two cases (Rabbits II and IV) and there was a rise in Rabbits I and II. The increased ammonia nitrogen was noticed mostly in cases where the urea nitrogen quotient showed decrease.

From the experiments an acceleration of protein metabolism is evident, followed by an increase of non-protein nitrogen in blood and an increased total nitrogen output in urine. This acceleration is attributed mainly to increased autolysis of tissues. Evidently, as shown by the examination of pathological changes of liver parenchyma, the progressive degeneration of liver cells is not always accompanied with marked changes in urea content in blood and urine. Our results are in harmony with those of Jackson (6), Marshall (5), and Chasatzky (11), as far as these investigators claim the relatively little change in urea formation in liver insufficiency.

I am including some brief clinical observations and protocols of autopsies and histological examinations of livers and kidneys.

The duration of poisoning, in spite of the same dose for each rabbit, is varying within broad limits; also the loss of body weight. In antimony poisoning the loss of weight is comparatively much greater. Icterus was noticed during last days of poisoning. In two rabbits anuria of short duration occurred, followed by albuminuria. In all cases of poisoning, rabbits showed diarrhea, the excreta being changed in color and consistency.

*Autopsy and Histological Changes in Livers and Kidneys of Rabbits Poisoned by Sodium Arsenite.*

*Rabbit 1.*—Gastrointestinal tract showed hemorrhages in stomach and small intestine; liver enlarged, of a dark brown color; kidneys without any macroscopical changes.

*Microscopical Examination.*—Liver: Slight congestion in the vessels and capillaries. No fatty changes in cells, nuclei fairly well stained. Kidneys: The vessels and capillaries were moderately congested. Convolted tubules were swollen.

*Rabbit 2.*—Hemorrhages in mucosa of stomach and small intestine. Liver showed bright yellow color at the margin of the lobes. Kidneys showed hemorrhages in the cortex.

*Microscopical Examination.*—Liver: Complete degeneration of parenchymal cells, mostly about the central veins. Fatty changes very evident. Kidneys: The degenerative changes in the glomeruli and tubules were very marked. The lumina of the tubules were filled with degenerated cells.

**Rabbit 3.**—Hemorrhagic changes in gastrointestinal tract. Liver of yellow color in peripheral parts of the lobes. Kidneys showed hemorrhages in the cortex.

*Microscopical Examination.*—Liver: The cells of the lobules were full of fat. Some cells showed only very slight degenerative changes. Kidneys: Degenerative changes in the tubules and glomeruli. The lumina obliterated with necrosed cells.

**Rabbit 4.**—Hemorrhages in stomach and small intestine. Liver atrophied; of yellow color. Kidneys showed hemorrhages in the cortex.

*Microscopical Examination.*—Liver: Fatty changes in parenchymal cells very marked. Only few cells in periphery of lobules without fat. Kidneys: Hemorrhages in cortex; congestion of wall of the glomeruli.

The microscopical examination showed no fatty degeneration of liver in Rabbit 1; in the other three rabbits the fatty changes were apparent; most severe destruction of liver cells was in Rabbit 2. The kidneys of Rabbit 1 showed a state of hyperemia, the other three rabbits showed marked glomerulonephritis. The chemical changes in blood and urine indicated some relation to the pathological changes in liver and kidneys. The increase of non-protein nitrogen in blood was most apparent in Rabbit 2, also the urea nitrogen in blood and urine in this rabbit was very low on the last day of the period of poisoning.

*Autopsy and Histological Reports of Changes in Liver and Kidneys of Rabbits Poisoned by Potassium Antimony Tartrate.*

**Rabbit I.**—Hemorrhages in stomach and small intestine. Liver atrophied, of slight yellow color in the margin of the lobes. Kidneys were without pathological changes macroscopically.

*Microscopical Examination.*—Liver: Parenchymal cells in the intermediary zone of the lobules contained fat; those at the periphery were in a state of beginning fatty degeneration. Kidneys: Slight congestion of tubules and glomeruli.

**Rabbit II.**—The same pathological changes in gastrointestinal tract as in Rabbit I. Liver slightly congested and atrophied. Kidneys showed hemorrhages in the cortex.

*Microscopical Examination.*—Liver: Fatty degeneration of cells in the intermediary zone of lobules marked. The peripheral cells without fat. Kidneys: The lumina of tubules obliterated with necrosed cells.

**Rabbit III.**—Hemorrhages in stomach and small intestine. Liver showed marked atrophy and yellow color. Kidneys showed hemorrhages in the cortex.

*Microscopical Examination.*—Liver: Parenchymal cells in a state of necrosis. Nuclei not much stained; fatty degeneration advanced. Kidneys: Congestion of glomeruli.

**Rabbit IV.**—Congestion with hemorrhages in stomach and small intestine. Liver showed slight atrophy, yellow color. Kidneys showed the periphery of the cortex lighter in color.

*Microscopical Examination.*—Liver: Parenchymal cells in the intermediary zone of the lobules contained fat; those of the periphery contained none. Kidneys: Glomeruli showed slight congestion.

#### SUMMARY.

Experimental subacute poisoning in four rabbits by sodium arsenite and in four rabbits by antimony potassium tartrate has been induced and the changes in ratio between urea nitrogen and non-protein nitrogen in blood, and between urea and ammonia nitrogen and total nitrogen in urine have been followed.

It has been found that there is an increase of non-protein nitrogen in blood after administration of both poisons. In arsenic poisoning the increase is more apparent. In arsenic poisoning there is a rise of non-protein nitrogen due to rise of urea nitrogen. The urea nitrogen quotient rises with the rise of urea nitrogen.

The rise of non-protein nitrogen and urea nitrogen in blood is associated with an increase of these constituents in the urine. The ammonia nitrogen quotient in the urine of poisoned rabbits seems to be inversely proportional to the urea nitrogen quotient.

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## DETOXICATION OF BENZOIC ACID IN MAN.

By J. L. BRAKEFIELD.

(From the Department of Biology, Howard College, Birmingham.)

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A review of the literature on the fate of benzoic acid in the animal body, the place of detoxication, and the manner in which it is detoxicated, indicates the fact that the workers are not wholly agreed.

Bunge and Schmeideberg (1) attempted to show that the kidney detoxicated benzoic acid. Kingsbury and Bell (2) and Lackner, Levinson, and Morse (3) did some work which indicates that the liver is involved in the detoxication of benzoic acid. Delprat and Whipple (4) made some experiments which give further evidence that the liver is normally concerned in the detoxication of benzoic acid. All these investigators assumed that the detoxication was effected by conjugation of the benzoic acid with glycine and excreted as hippuric acid.

Recent experiments indicate that benzoic acid is detoxicated in at least two ways in the dog and the pig. Thus Csonka (5) has shown that benzoic acid when fed to the pig is eliminated in the urine partly as free benzoic acid, partly as hippuric acid, and the remainder as benzoyl glucuronic acid. Brakefield and Schmidt (6) have shown that in the dog benzoic acid is detoxicated in at least two ways: (a) by conjugation with glycine and (b) by combining with glucuronic acid.

In light of the experiments which have been carried out on the dog and the pig it appeared of interest to determine the distribution of the detoxication of benzoic acid in man. Subjects were selected who felt no ill effects from the ingestion of sodium benzoate in rather large quantities. Doses of sodium benzoate which would yield 5 to 6 gm. of benzoic acid were taken.

### EXPERIMENTAL.

The experiments reported in this paper were carried out on human subjects. Urine samples from the subjects were found to

contain little or no benzoic acid or reducing substances. The subjects were found to be able to detoxicate 5 to 6 gm. of benzoic acid in 10 hours. The distribution of the excreted benzoic acid was determined by the methods outlined below.

### *Analytical Methods.*

The free benzoic acid was determined by the method of Delprat and Whipple (4), the total benzoic acid by the method described by Kingsbury and Swanson (7). The difference between the total and free benzoic acid gives the amount of conjugated benzoic acid. The glucuronic acid was determined by the method employed by Csonka (5) except that the Shaffer-Hartmann reducing agent

TABLE I.

Subject.	Benzoic acid.	Time of collection.	Benzoic acid as hippuric acid.	Benzoic as benzoyl glucuronic acid.	Benzoic acid, free.
	<i>gm.</i>	<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
A	5	10	4.85	00	0.1
	6	10	5.66	00	0.25
	5	10	4.72	00	0.18
B	5	10	4.85	00	0.05
	5	10	4.78	00	0.14
	5	10	4.90	00	0.00

rather than the Benedict reagent was employed. The total benzoic acid combined, less that which was combined with glucuronic acid (benzoyl glucuronic acid), gives the amount of benzoic acid which was combined with glycine (hippuric acid).

### *Urine Samples.*

The bladder was completely emptied at the outset of the experiment and the urine collected for a period of 10 hours. The urine was collected in a bottle which contained approximately 50 cc. of a 2 per cent solution of sulfuric acid to prevent the breaking down of the conjugated acid. The subjects were on an ordinary diet.

*Results.*

The results obtained from the experiments are recorded in Table I and show the amounts of combined and free benzoic acid and the distribution of the combined benzoic acid. The results show that in the two subjects in question approximately 5 gm. of benzoic acid were detoxicated by being combined with glycine and excreted as hippuric acid. In no case was there a detectable amount of benzoyl glucuronic acid. This is contrary to that which may have been expected in light of recent experiments on dogs, pigs, and sheep.

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# THE RELATIONSHIP BETWEEN THE STRUCTURE AND THE BIOLOGICAL ACTION OF THE CARDIAC GLUCOSIDES.

BY WALTER A. JACOBS AND ALEXANDER HOFFMANN.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

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The probable close relationship in chemical structure of certain substances of glucosidic nature which exhibit a characteristic cardiac or so called digitalis action has been generally recognized.<sup>1</sup> In work from this laboratory special consideration has been given to the fact that a number of the aglucones of these glucosides give reactions with sodium nitroprusside, a property which was shown to be due to an association of the lactone group with a double bond, as  $\Delta^{\beta,\gamma}$  lactones in which the double bond may be within or without the lactone ring.<sup>2</sup> This fact was coupled with observations on the effect of hydrogenation of the double bond on the toxicity of several of these substances. Windaus, Bohne, and Schwieger<sup>3</sup> found that hydrogenation of digitalinum verum converts it into dihydrodigitalin, a "physiologically completely inactive substance." Similarly, a few crude toxicity tests on frogs by ourselves with dihydroouabain showed that a great reduction in toxicity was produced and since the material prepared by us had been obtained only as an amorphous substance, the possibility was considered that the observed persistence of any digitalis action in this case may have been due to contamination with very small amounts of unhydrogenated ouabain. More recently Cloetta<sup>4</sup> has similarly concluded that hydrogenation

<sup>1</sup> Lehmann, E., *Arch. Pharm.*, 1897, ccxxv, 175.

<sup>2</sup> Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1926, lxxvii, 333. Jacobs, W. A., Hoffmann, A., and Gustus, E. L., *J. Biol. Chem.*, 1926, lxx, 1.

<sup>3</sup> Windaus, A., Bohne, A., and Schwieger, A., *Ber. chem. Ges.*, 1924, lvii, 1388.

<sup>4</sup> Cloetta, M., *Arch. exp. Path. u. Pharmacol.*, 1926, cxii, 324.

destroys the cardiac action in the case of several digitalis aglucones which were isolated by him. One of these, which he has termed digitaligeninum cristallisatum, is identical with gitoxigenin and therefore closely related to digitalinum verum which Windaus and Schwarte<sup>5</sup> have shown to be most probably a gitoxigenin glucoside. The opinion was expressed by us that this association of the lactone group and the double bond may be essential for the cardiac action of this general group of substances.

Lehmann<sup>1</sup> was the first to isolate from *Periploca græca* an active crystalline glucoside periplocin which yielded on hydrolysis, periplogenin. In the course of investigations on the correct formulation, etc., of periplogenin, which will be published subsequently, we have found that this substance is a lactone and also gives the nitroprusside reaction. Similarly, antiarin is a lactone as may be surmised from its behavior towards alkali as reported by Kiliani.<sup>6</sup> A sample of this substance was very kindly presented to us by Professor Magnus of the University of Utrecht, and it has been found to give the Legal reaction with nitroprusside. It appears probable, therefore, that these substances belong also to the group of  $\Delta^{\beta,\gamma}$ -lactones. On the other hand, both scillarin and its aglucone, scillaridin,<sup>7</sup> from *Scilla maritima* or *Urginea scilla*, failed to give this reaction. A crude, amorphous but highly active substance which we have isolated in a preliminary study of the active substances of *Convallaria majalis* did not give a positive nitroprusside test. A similar failure has already been recorded in the case of the bufagin isolated by Abel and Macht from the tropical toad, *Bufo aqua*.<sup>8</sup> It appeared, therefore, that even if all of the naturally occurring substances which exhibit an intense digitalis action can be considered in the same general structural category, they are not necessarily all  $\Delta^{\beta,\gamma}$ -lactone derivatives. This raised again the question as to whether the cardiac action of substances which are  $\Delta^{\beta,\gamma}$ -lactones is really

<sup>5</sup> Windaus, A., and Schwarte, G., *Ber. chem., Ges.*, 1925, lviii, 1516.

<sup>6</sup> Kiliani, H., *Arch. Pharm.*, 1896, ccxxxiv, 449; *Ber. chem. Ges.*, 1910, xliii, 3577.

<sup>7</sup> The samples of scillarin and scillaridin prepared by the Sandoz Chemical Works of Switzerland were generously presented to us by the H. A. Metz Laboratories.

<sup>8</sup> Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1926, lxxvii, 338.

attributable in character to this structural feature or whether it is a property inherent in the molecule as a whole which may be affected in intensity by the double bond properly allocated in the molecule.

We have approached this problem again and have repeated and extended the study of the effect of hydrogenation on the toxicity of these glucosides. Fortunately, cymarín on hydrogenation yielded a beautifully crystalline and certainly homogeneous dihydro derivative. This substance retained a potent digitalis action for frogs, although this was somewhat less than 5 per cent of that exhibited by cymarín itself. Similarly, the preparation and study of dihydroouabain was repeated and although this substance could be obtained only in amorphous form, the hydrogenation was performed under conditions which made certain the complete transformation of the ouabain employed. The dihydro glucoside was found to retain a marked digitalis action although the effective dose was again greatly diminished and only about 7 per cent of that of ouabain itself.

In the experiments of Windaus, Bohne, and Schwieger,<sup>3</sup> the non-toxic character of even 8 mg. of dihydrodigitalin for a 47 gm. frog was shown, whereas 0.5 mg. of digitalinum verum proved fatal for a 35 gm. frog. The amount of the former used, however, was only 12 times per gm. of frog of the amount of the latter, and it is still possible that a larger dose of the hydro derivative if practicable might have shown a definite digitalis action. Owing to the lack of the necessary material we have not been able to test this point ourselves. The same consideration may possibly hold for the so called dihydro- and tetrahydrodigitaligenin of Cloetta where the toxicities of the unhydrogenated "genins" were of themselves not of the highest order. Since digitoxin is the most toxic of the digitalis glucosides, it was thought possible that if the hydrogenated substance retained any inherent digitalis action the dose in this case might be practicable for administration. Although digitoxin yielded a beautifully crystalline dihydro compound, the latter proved, however, to be too insoluble in all suitable solvents to permit such concentrations to be tested by the ordinary frog method which might exhibit an action. At any rate, the results with cymarín and ouabain permit the definite conclusion that the biological action of the cardiac glucosides



belonging to the group of unsaturated lactones is therefore a property apparently inherent in the molecule as a whole and the double bond alone does not determine the character but may contribute to the intensity of this action.

To make certain of the nature and homogeneity of the dihydro derivative obtained by hydrogenation of cymarín, the former was hydrolyzed by dilute acid. Although dihydrostrophanthidin was readily obtained, a sparingly soluble by-product was also isolated which was readily shown to be dihydromonoanhydrostrophanthidin produced by the secondary elimination of 1 mol of water from dihydrostrophanthidin, since this product was readily obtained under the same conditions from dihydrostrophanthidin itself. In this respect dihydrostrophanthidin presents an interesting greater lability towards hydrochloric acid than strophanthidin. The structural significance of this property will be considered in a subsequent communication.

#### EXPERIMENTAL.

*Hydrocymarín*.—3.5 gm. of cymarín (containing 1 CH<sub>3</sub>OH) were hydrogenated in dilute methyl alcoholic solution with 0.45 gm. of colloidal palladium. After 24 hours the absorption of approximately 1 mol of H<sub>2</sub> was complete. The palladium was removed with colloidal ferric hydroxide solution and the filtrate was concentrated under diminished pressure. A solution of the residue in 10 cc. of methyl alcohol was diluted with 20 cc. of warm water. On cooling, well formed stout rhombs crystallized. After two recrystallizations, the yield was 2.8 gm. The substance sintered at 121° and melted with effervescence at 128°, solidifying again to a mass which melted again with effervescence at 190°. It is readily soluble in alcohol, chloroform, and acetone, and but sparingly so in water although a solution in the latter is readily prepared by dilution of a solution of the substance in a few drops of alcohol. Contrary to cymarín, it no longer gives a nitroprusside reaction. For the anhydrous substance  $[\alpha]_D^{22} = +17.8^\circ$  ( $c = 1.11$  in pyridine).

*Air-Dry Substance*. Dried at 100° and 15 mm.

C<sub>30</sub>H<sub>46</sub>O<sub>9</sub>·H<sub>2</sub>O. Calculated. H<sub>2</sub>O 3.13.

Found. " 3.73.

*Anhydrous Substance.*

$C_{30}H_{46}O_8$ . Calculated. C 65.41, H 8.43.  
Found. " 65.32, " 8.48.

A solution of 1 gm. of dihydrocymarin in 8 cc. of 50 per cent methyl alcohol was treated with 2 cc. of hydrochloric acid (1.19) and then left at room temperature for 5.5 hours. After dilution with 60 cc. of water it was left at 0° for 18 hours, during which a crust of crystals separated. After collection this fraction (0.12 gm.) was recrystallized from 18 cc. of hot methyl alcohol. Long needles separated which melted at 224° after preliminary sintering at 215°.  $[\alpha]_D = +48.8^\circ$  ( $c = 1.23$  in pyridine). This substance is therefore identical with dihydromonoanhydrostrophanthidin, which will be described below.

The above crude hydrolysis mother liquor was neutralized to litmus with  $NaHCO_3$  solution. Stout, highly refracting prisms separated. After collection (0.33 gm.) it was recrystallized from dilute methyl alcohol forming prisms which melted at 105° after preliminary sintering and gave an  $[\alpha]_D = +42^\circ$  ( $c = 1.38$  in pyridine). The substance was therefore dihydrostrophanthidin. Saturation of the crude aqueous mother liquor with ammonium sulfate gave an additional 0.19 gm. of dihydrostrophanthidin.

*Dihydromonoanhydrostrophanthidin.*—4.3 gm. of dihydrostrophanthidin were dissolved in 10 cc. of methyl alcohol and the solution was treated with a mixture of 7.5 cc. of water and 2.5 cc. of HCl (1.19). When heated in the water bath under a reflux, the clear solution became cloudy and suddenly set to a thick crystalline pap. After 30 minutes in all, the mixture was cooled and the crystals were collected and washed with cold methyl alcohol. Recrystallization of the dried substance was accomplished by dissolving in 35 cc. of 50 per cent acetic acid and by diluting the filtrate with 15 cc. of hot water. On cooling long needles separated which sintered regularly at 218° and melted at 225°. The yield was 2.7 gm. For further purification the substance was recrystallized either from acetone or by dilution of the warm concentrated solution in chloroform with dry ether. In this manner the melting point was raised to 232° after sintering at 226°.

The substance is easily soluble in chloroform, and relatively

sparingly so in acetone and warm alcohol. It is almost insoluble in ether.  $[\alpha]_D^{26} = +48.5^\circ$  ( $c = 1.978$  in pyridine).

$C_{28}H_{42}O_5$ . Calculated. C 71.08, H 8.31.

Found. (a). " 71.22, " 8.23.

(b). " 70.97, " 8.21.

*Dihydroouabain*.—A solution of 2 gm. of ouabain in 150 cc. of water was hydrogenated in the presence of 0.4 gm. of colloidal palladium. After 5 hours the absorption of hydrogen stopped at approximately 1 mol. The solution was saturated with ammonium sulfate which caused precipitation of the amorphous glucoside together with the catalyst. The collected precipitate was dried and then repeatedly extracted with cold absolute alcohol. The combined extracts containing the dihydro compound were concentrated to 10 cc. under diminished pressure. Careful addition of dry ether produced in this solution a precipitation of amorphous material which was completed with petroleic ether. After collection 1.4 gm. of a colorless, amorphous unhygroscopic powder were obtained which was very easily soluble in the usual hydroxyl-containing solvents. In spite of many attempts it could not be obtained in crystalline form. In the form obtained it softened under  $100^\circ$  and foamed up at  $105^\circ$ . Whereas 1 mg. of ouabain itself gave a strong Legal reaction, 28 mg. of the dihydro compound dissolved in 1 cc. of water gave a completely negative reaction.  $[\alpha]_D^{30} = -47.4^\circ$  ( $c = 2.826$  in water for the anhydrous substance).

$C_{30}H_{48}O_{12}$ . Calculated. C 59.96, H 8.06.

Found. " 60.08, " 8.26.

*Dihydrodigitoxin*.—For this preparation a digitoxin was used which had been purified by the method recommended by Cloetta<sup>9</sup> and melted sharply at  $240^\circ$ . The substance was anhydrous and a micro combustion gave the following figures. 0.35 gm. of this substance was hydrogenated in dilute methyl alcoholic solution with 0.2 gm. of colloidal palladium. In the course of 16 hours 15 cc. of hydrogen were absorbed. Calculated for 1 mol,  $H_2$  is about 10.2 cc. for the formula  $C_{41}H_{64}O_{13}$  (Windaus).<sup>10</sup>

<sup>9</sup> Cloetta, M., *Arch. exp. Path. u. Pharmacol.*, 1920, lxxxviii, 133.

<sup>10</sup> Windaus, A., *Nachr. Ges. Wissensch. Goettingen, Math.-Physik. Klasse*, 1926, 170.

The palladium was removed by treatment with a minimal amount of colloidal ferric hydroxide solution. The filtrate on concentration yielded glistening needles which were recrystallized twice from dilute methyl alcohol. Contrary to digitoxin itself, the substance gave no reaction with alkaline sodium nitroprusside solution. It melted at 202–204° and showed a very weak dextro-rotation.  $[\alpha]_D^{25} = +2.4^\circ$  ( $c = 0.622$  in pyridine). Dihydrodigitoxin exhibits about the same solubilities as digitoxin but appears to be less soluble than this substance in dilute solvents. At any rate, on dilution of its alcoholic solution with water it crystallizes

TABLE I.

Substance.	Toxicity in frog units of 1 gm. air-dry substance.	Toxicity in frog units of 1 gm. anhydrous substance.	Ratio of toxic- ity of the glu- coside to its hydro deriva- tive.
Cymarín.....	1,630,000	1,725,000	23.3 : 1
Dihydrocymarín.....	72,000	74,000	
Ouabain.....	2,040,000	2,494,000	16.1 : 1*
Dihydroouabain.....	155,000	155,000	

\* In our earlier paper (Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1926, lxvii, 338), we reported the toxicity of dihydroouabain as much lower on the basis of a few crude preliminary tests. The present figures we believe are correct since they are the mean of numerous determinations.

too quickly to permit of its injection into frogs in sufficiently strong concentration.

#### *Comparative Toxicity Determinations on Frogs.*

For the toxicity determinations the method employed was based essentially on that recommended by the Second International Conference on the Biological Standardization of Certain Remedies as published in *Public Health Reports*,<sup>11</sup> for digitalis. Frogs, *Rana pipiens*, of about 40 gm., were kept under constant conditions at room temperature before use. The solutions were injected into the breast lymph sac in amounts up to 0.5 cc. The

<sup>11</sup> *Pub. Health Rep., U. S. P. H.*, 1926, xli, 505.

frogs were observed for signs of intoxication for 4 hours when in case of survival they were held for 18 hours longer for observation.

The solutions of cymarín and dihydrocymarín were prepared by diluting the concentrated alcoholic solution so that the concentration of alcohol was in each case respectively 8 and 5 per cent. Ouabain and dihydroouabain were dissolved in water alone. The figures given in Table I are the mean values of numerous experiments.

## STROPHANTHIN.

### XI. THE HYDROXYL GROUPS OF STROPHANTHIDIN.

By WALTER A. JACOBS AND EDWIN L. GUSTUS.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, July 2, 1927.)

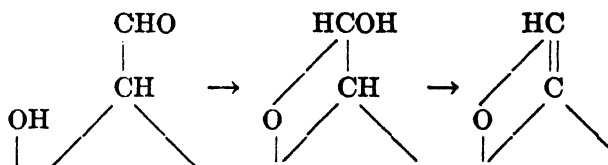
In the present and immediately following communications on strophanthidin, the more recent developments in the study of its structure and the relation to this substance of a number of its previously reported derivatives will be presented. Strophanthidin has been shown to be a saturated tetracyclic trihydroxy- $\Delta^{\beta,\gamma}$ -lactone aldehyde.<sup>1</sup> By the usual methods of acylation, only one of the hydroxyl groups has been directly acylated. This suggested the possible tertiary character of the remaining hydroxyls, which was supported by their greater lability. The successive removal of one (which will be called OH<sup>I</sup>) and then of a second hydroxyl group (OH<sup>II</sup>) was accomplished in absolute alcoholic hydrogen chloride with the formation of double bonds,  $\Delta_1$  and  $\Delta_2$ , respectively. At the same time, the aldehydic group was converted into the ethylhalfacetal which then lost water with the acylatable hydroxyl (OH<sup>III</sup>) to form an oxidic structure.<sup>2</sup> These substances, the ethylals of oxidomonoanhydro- and oxidodianhydrostrophanthidin, respectively, were readily converted on hydrolysis with acid into the corresponding hydroxy aldehydes. The facility with which this oxidic structure was formed, as well as the fact that dianhydrostrophanthidin itself mutarotated in solution and reacted in either the oxidic or aldehydic form, suggested that the acylatable hydroxyl (OH<sup>III</sup>) is  $\gamma$  or  $\delta$  to the aldehyde group. Finally, the removal of a molecule of water from the oxidic form of dianhydrostrophanthidin gave rise to

<sup>1</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lix, 713; 1925, lxiv, 383; 1925, lxv, 491.

<sup>2</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lix, 713.

trianhydrostrophanthidin<sup>3</sup> which no longer contains a carbonyl or hydroxyl group. Contrary to dianhydrostrophanthidin, the double bonds,  $\Delta_1$  and  $\Delta_2$ , respectively, formed by the removal of  $\text{OH}^I$  and  $\text{OH}^{II}$ , no longer absorbed bromine and could not be hydrogenated with palladium. This suggested that the loss of water in the formation of trianhydrostrophanthidin gives rise to a new double bond,  $\Delta_3$ , which forms a conjugated system of three double bonds with  $\Delta_1$  and  $\Delta_2$  or a benzenoid structure. Further substantiation of this view will be presented in the following paper on the behavior of trianhydrostrophanthidin towards oxidizing agents.

Since the carbonyl group of strophanthidin is aldehydic and not ketonic in character, as originally assumed, it must be adjacent to a CH group,



in order to make possible the loss of water from the oxidic form of dianhydrostrophanthidin to form a new double bond which then may shift its position into the ring containing  $\Delta_1$  and  $\Delta_2$ . Facts which have accumulated appear to fit best with the conclusion that the aldehydic group is attached directly to a CH group contained in a six-membered ring containing  $\text{OH}^I$  and  $\text{OH}^{II}$ .

Further evidence regarding the allocation and the nature of the hydroxyls of strophanthidin has now been obtained by a study of the methyl ester of the acid  $\text{C}_{23}\text{H}_{32}\text{O}_7$ , *strophanthidinic acid*,<sup>4</sup> which is formed on oxidation of the aldehyde group of

<sup>3</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lxiii, 123.

<sup>4</sup> Although the term strophanthidinic acid might be applied to the acid obtained by the saponification of strophanthidin, as already suggested (the term isostrophanthidinic acid was suggested for saponified isostrophanthidin, Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lxi, 388), we have adopted this term rather for its oxidation product and wish to withdraw the former suggestion. The adoption of suitable terminology is extremely difficult and must be arbitrary in work of this kind where the number of complex substances soon becomes large and the relationships among them so intricate. Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 556.

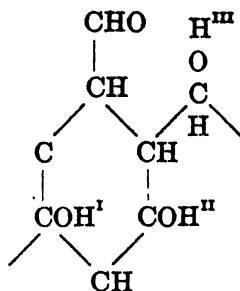
strophanthidin to carboxyl.<sup>5</sup> This ester, as previously described, forms a monobenzoate like strophanthidin. On oxidation with chromic acid, the ester is smoothly converted into a keto ester,  $C_{24}H_{32}O_7$ , *strophanthidonic methyl ester*, which forms an oxime but no longer yields a benzoate.  $OH^{III}$  of strophanthidin is therefore secondary. The keto ester now displays a new property, in that one of the remaining hydroxyls has become very labile. On standing at ordinary temperature with dilute mineral acid, or more quickly when heated, water is split off with the formation of an unsaturated ketone. As we shall see, this hydroxyl in the original ester cannot be removed under similar conditions. Its lability, therefore, must depend upon the proximity of the carbonyl group. This suggests that the substance is a  $\beta$ -hydroxyketone which loses water to form a  $\Delta^{\alpha,\beta}$ -ketone. The olefinic character of the latter was shown by hydrogenation.

If the strophanthidinic methyl ester itself is treated with hot dilute acid, it is converted with the loss of 1 mol of water into monoanhydrostrophanthidinic methyl ester. In this case the hydroxyl group involved is different from the labile hydroxyl of the above hydroxyketone. This was directly shown by the oxidation of the monoanhydro ester with chromic acid to an intermediate monoanhydro keto ester in which the remaining hydroxyl was now readily removed with the formation of a dianhydro keto ester. Therefore this last hydroxyl, which comes under the influence of the newly formed carbonyl group, must be the same in both strophanthidinic methyl ester and its monoanhydro derivative.

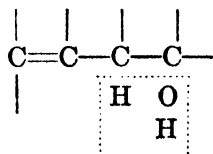
The loss of water in the case of strophanthidinic methyl ester is analogous to the formation of monoanhydrostrophanthidin (ethylal) from strophanthidin, and the hydroxyls involved in each case are the same, namely  $OH^I$ .  $OH^{II}$  is situated on the same ring as  $OH^I$  and also  $\beta$  to  $OH^{III}$ , which must in turn be attached to a carbon atom of an adjoining ring. This may be seen by reference to the accompanying graphic representation.

<sup>5</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxx, 494.





The previously reported conversion of the ethylal of oxidomonoanhydrostrophanthidin into the ethylal of oxidodianhydrostrophanthidin must depend upon a similarly conditioned lability of OH<sup>II</sup>. In this case, this is induced not by a carbonyl group but by the fact that the double bond  $\Delta_1$  already is, or may first shift to, a position  $\beta, \gamma$  to OH<sup>II</sup> and so render the latter more labile.



It is hoped to present a complete picture of the various phases involved in this case at a later date.

The above results have made necessary a reconsideration of the conclusions drawn from previous work. In a former communication,<sup>6</sup> a pseudostrophanthidin formed by the action of strong hydrochloric acid on strophanthidin was described, which was shown to be a stable oxidic form of the hydroxyaldehyde. In analogy with dianhydrostrophanthidin, it was assumed that the same hydroxyl was involved in the oxidic structure in both cases, namely OH<sup>III</sup>, which, as we have seen, is secondary. Similarly, when strophanthidinic acid was dissolved in strong hydrochloric acid, it was converted into a stable neutral dilactone, which we wish now to designate as *strophanthidinic lactone*. This substance was shown to possess the same oxidic ring as pseudostrophanthidin, since oxidation of either substance with chromic acid yielded the same keto dilactone. If the secondary

<sup>6</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxxiii, 131; 1925, lxxv, 495.

hydroxyl ( $\text{OH}^{\text{III}}$ ) were involved in these oxidic forms, then the new keto group would have been formed from another secondary hydroxyl. But if such were the case, strophanthidinic methyl ester should yield a diketone on oxidation. Since only a monoketone has been obtained, the only alternative is that the ketonic group of the keto dilactone is produced from  $\text{OH}^{\text{III}}$ , as in the previous case, and that the formation of the oxidic ring of pseudostrophanthidin and the dilactone involves either  $\text{OH}^{\text{I}}$  or  $\text{OH}^{\text{II}}$ . This has now been substantiated by the fact that a benzoate can still be obtained from the dilactone, which shows the presence of  $\text{OH}^{\text{III}}$  as such in this substance. There is also no longer reason, as once expressed, to consider that  $\text{OH}^{\text{I}}$  and  $\text{OH}^{\text{II}}$  may be other than tertiary. The choice between  $\text{OH}^{\text{I}}$  and  $\text{OH}^{\text{II}}$  as the group involved in the oxidic structure was determined in favor of the former, since the condition necessary for the formation of pseudostrophanthidin and the dilactone is the action of strong hydrochloric acid. Even under conditions which were more severe than those used for the production of the monoanhydro compound from its methyl ester, strophanthidinic acid and its dihydro derivative form neutral dilactones and no monoanhydro derivatives.  $\text{OH}^{\text{I}}$  must, therefore, be protected by this ring closure, which apparently occurs before there is opportunity for the loss of this hydroxyl by removal as water.

The properties of pseudostrophanthidin and the dilactone have shown them to be stable oxidic structures, and it appeared necessary to postulate as a possibility a preliminary rearrangement of groups under the influence of the reagent during their formation. This was confirmed by a comparative experiment in which dihydrostrophanthidinic acid, which was used because it is more stable to alkali after saponification of its lactone group than the unsaturated strophanthidinic acid, and the dihydrodilactone obtained from it by the action of strong acid were heated under identical conditions; that is, for 3 hours, with 2 per cent alkali. If the latter had been obtained from the former by simple lactonization without previous rearrangement, the resulting dibasic acid should have been identical in each case and on gentle acidification should have yielded the same lactone acid, namely dihydrostrophanthidinic acid, provided, of course, no rearrangement of another type had been produced by the alkali. Whereas in the former case dihydro-

strophanthidinic acid (m.p. 132–134°) was recovered unchanged, in the case of the dilactone a rapidly lactonizing acid was formed which gave the original neutral dilactone (m.p. 227–229°). In dihydrostrophanthidinic acid, and therefore in strophanthidin, the carboxyl and aldehyde groups are *trans*, respectively, to OH<sup>I</sup>, whereas concentrated acid causes rearrangement to the *cis* form with subsequent production of the oxidic structure exhibited by pseudostrophanthidin and the dilactones. It appears probable that the carbon atom carrying OH<sup>I</sup> is the center of asymmetry involved in this rearrangement, although with the facts available this can at the moment be only conjectural. In the case of strophanthidinic methyl ester, the carboxyl group is protected and such an oxidic structure is not possible. Therefore, only loss of water can occur with the formation of an unsaturated anhydro ester.

The stability of the second lactone group in the dilactones, as shown by its much greater resistance to the saponifying action of dilute alkali than that displayed either by the original  $\Delta^{\beta,\gamma}$ -lactone group or by its dihydro derivative, has now been found to be a general property of the esters of this particular carboxyl, which require more vigorous treatment with alkali for saponification. Similar observations have been made in the isostrophanthidin series, as presented in the following communications.

#### EXPERIMENTAL.

*Strophanthidonic Methyl Ester*.—4.5 gm. of strophanthidinic methyl ester were dissolved in 70 cc. of acetic acid. After chilling below 15°, 15 cc. of chromic acid solution (400 gm. of water, 80 gm. of sulfuric acid, and 53 gm. of chromic acid) were added. The reaction was prompt. The mixture was kept around 10° for  $\frac{1}{2}$  hour and then diluted to a liter and saturated with ammonium sulfate. The turbid solution gradually crystallized when rubbed. The substance was collected with dilute ammonium sulfate solution and finally washed with a little water. The yield of the ketone was excellent, and in the reaction mixture the excess of chromic acid remained unused.

The substance was recrystallized either from hot water, in which it is appreciably soluble, or by diluting a concentrated solution in methyl alcohol with 3 volumes of water. It separated

as a crust of minute, short, stout prisms which melted at 161–162°. It is easily soluble in acetone and in the hydroxyl-containing organic solvents.

$$[\alpha]_D = +26^\circ \text{ (c = 1.03 in pyridine).}$$

*Air-Dry Substance.* Dried at 100° and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{24}\text{H}_{22}\text{O}_7 \cdot \frac{1}{2}\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  2.08.

Found. " 2.09.

*Anhydrous Substance.*

$\text{C}_{24}\text{H}_{22}\text{O}_7$ . Calculated. C 66.63, H 7.46.

Found. " 66.64, " 7.34.

In an attempt to benzoylate this substance in pyridine solution with benzoyl chloride, the ester was recovered unchanged. It formed, however, an oxime, but the reaction was apparently complicated by partial loss of water with the formation of the oxime of the following monoanhydroketone. Since a satisfactory oxime was obtained from the latter, as described below, no further attempt was made to purify the oxime obtained from strophanthidonic ester.

*Monoanhydrostrophanthidonic Methyl Ester.*—For this preparation, crude strophanthidonic methyl ester was used just as it is obtained by dilution of the preceding oxidation mixture. 1.5 gm. were refluxed for 15 minutes in a mixture of 20 cc. of methyl alcohol and 5 cc. of 10 per cent hydrochloric acid. When diluted and cooled, the anhydro ester crystallized at once. After collection with dilute alcohol, the yield was 1.2 gm. Recrystallized from dilute methyl alcohol, it formed small, glistening, stout prisms or rhombs which melted at 210°, although the melting point fluctuated considerably, with different preparations or following different modes of recrystallization, between 203° and 213°. It is easily soluble in chloroform and less readily so in descending order in acetone, alcohol, and benzene.

$$[\alpha]_D = +123^\circ \text{ (c = 1.107 in pyridine).}$$

Attempts to benzoylate the substance resulted only in recovery of the unchanged material.

*Anhydrous Substance.* Obtained by drying at 100° and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{24}\text{H}_{20}\text{O}_6$ . Calculated. C 69.53, H 7.30.

Found. " 69.49, " 7.17.

*Monoanhydrostrophanthidonic Methyl Ester Oxime*.—The oxime was obtained by heating the ester with hydroxylamine hydrochloride and sodium acetate in methyl alcoholic solution for several hours. On dilution, the substance easily crystallized. From methyl alcohol, it formed small, short prisms which were very sparingly soluble in methyl and ethyl alcohols and acetone. It melted with decomposition at 290–291°.

$C_{24}H_{31}O_6N$ . Calculated. C 67.09, H 7.28.  
Found. " 67.00, " 7.07.

*Dihydromonoanhydrostrophanthidonic Methyl Ester (Desoxystrophanthidonic Methyl Ester)*.—1 gm. of monoanhydrostrophanthidonic methyl ester dissolved in 200 cc. of methyl alcohol was hydrogenated with 0.1 gm. of colloidal palladium. After 20 minutes, rapid absorption ceased at the 1 mol stage. The mixture was concentrated to dryness and the residue was exhausted with chloroform. The glassy residue from the chloroform extract readily crystallized in a small volume of methyl alcohol as small rhombs and prisms which melted at 145–147° and contained solvent of crystallization, since it resolidified and then melted again at 207–209°. When allowed to stand in a desiccator over calcium chloride, the melting point rose to 209°. The substance appeared to separate with different amounts of solvent of crystallization, since a sample when freshly obtained from methyl alcohol melted at 100–105°, resolidified, and then melted at 209°. It still gave the Legal test, showing that the  $\Delta^{\beta,\gamma}$  double bond was not hydrogenated. For analysis, it was first dried in a desiccator and then at 100° and 15 mm. over  $H_2SO_4$ .

*Anhydrous Substance*.

$C_{24}H_{32}O_6$ . Calculated. C 69.19, H 7.75.  
Found. " 69.29, " 7.76.

The same substance was obtained by hydrogenation in acetic acid solution with palladium black.

*Monoanhydrostrophanthidinic Methyl Ester*.—When strophanthidinic methyl ester was heated with dilute methyl alcoholic hydrochloric acid under the conditions used for the above keto ester, most of it was recovered unchanged. More drastic conditions were found necessary, which, however, somewhat affected the yield.

3 gm. of strophanthidinic methyl ester were refluxed in a mixture of 30 cc. of methyl alcohol and 3 cc. of concentrated hydrochloric acid (1.19) for 2 hours. On careful dilution and rubbing, the anhydro derivative slowly crystallized. The process was aided by careful subsequent dilution. Recrystallized from dilute methyl alcohol, it formed rosettes or sheaves of delicate needles which melted at 205–206°. The yield was 1.5 gm. It is readily soluble in methyl and ethyl alcohols, acetone, and chloroform, and very sparingly soluble in ether. In sulfuric acid, it dissolves with a gradually deepening brown color which finally changes to a brown-red.

$$[\alpha]_D^{25} = +25^\circ \text{ (c = 1.01 in pyridine).}$$

$C_{24}H_{32}O_6$ .	Calculated.	C 69.19, H 7.75.
	Found.	" 69.60, " 7.75.

The ester group of this substance is much more resistant to alkali than the lactone group.

0.812 gm. of substance was refluxed for 2 hours in 10 cc. of alcohol and 10 cc. of 0.1 N NaOH and then titrated back against phenolphthalein. Calculated for 1 equivalent, 1.81 cc. Found, 2.18 cc.

0.4003 gm. was refluxed for 1½ hours in 20 cc. of alcohol and 20 cc. of 0.1 N NaOH. Calculated for 1 equivalent, 8.94 cc. Found, 10.14 cc.

These titrations show that only a small fraction of the ester group was affected by these conditions. When the above titration mixtures were acidified to Congo red, resinous material separated and no crystalline substance could be isolated. Titration experiments with strophanthidinic methyl ester gave similar results.

0.0964 gm. of anhydrous ester consumed 2.44 cc. of 0.1 N NaOH. Calculated for 1 equivalent, 2.22 cc. Reacidification caused relactonization but with the formation of a crystalline iso compound (see subsequent paper).

*Dianhydrostrophanthidonic Methyl Ester.*—0.5 gm. of the above monoanhydro ester was dissolved in 10 cc. of acetic acid, and the solution was then treated with 1.2 cc. of chromic acid solution. The reaction was prompt. After about 15 minutes, an equal volume of water was added, followed by 5 cc. of 10 per cent sulfuric acid. The latter was added for conversion into the dian-

hydro compound. On standing several days at ordinary temperature, the mixture slowly deposited a crust of crystals which were collected with water. Recrystallized by concentrating the solution in hot methyl alcohol, it formed lustrous, six-sided tablets which melted at 202–203°. Examination showed that this was the desired dianhydro compound which was formed by subsequent loss of water from the more soluble intermediate monoanhydro-strophanthidonic ester. It is readily soluble in chloroform and acetone, and less easily in the alcohols.

$$[\alpha]_D = +131^\circ \text{ (c = 1.047 in pyridine).}$$

$$\text{C}_{24}\text{H}_{26}\text{O}_6. \text{ Calculated. C 72.69, H 7.12.}$$

$$\text{Found. " 72.58, " 7.01.}$$

It was expected that in possible analogy with the case of saponifiability of the dianhydrodilactone  $\text{C}_{23}\text{H}_{26}\text{O}_4$ ,<sup>7</sup> and in contrast with that of the tetrahydrodilactone, dianhydrostrophanthidonic methyl ester should also be easily saponified. The ester group, however, proved to be still relatively resistant, so that besides the lactone group only a fraction of the ester was saponified under the conditions usually used.

0.0644 gm. of substance, when boiled for 2 hours in 10 cc. of alcohol and 10 cc. of 0.1 N NaOH, consumed 2.13 cc. Calculated for 1 equivalent, 1.63 cc.

*Dihydrostrophanthidinic Lactone Benzoate.*—The dihydrodilactone in dry pyridine solution was treated with benzoyl chloride. After 5 hours at 20°, the mixture was poured into dilute sulfuric acid. The precipitated oil was extracted with chloroform, which was in turn washed with water, carbonate solution, and then dried. Concentration yielded the benzoate which was collected with methyl alcohol. Recrystallized from acetone, it formed leaflets which melted at 271–272°.

$$\text{C}_{30}\text{H}_{30}\text{O}_7. \text{ Calculated. C 70.83, H 7.14.}$$

$$\text{Found. " 71.11, " 7.05.}$$

*Strophanthidinic Lactone Benzoate.*—The dilactone  $\text{C}_{23}\text{H}_{30}\text{O}_6$  was benzoylated as in the case of the dihydro derivative. From acetone, it formed hexagonal platelets which melted at 263–264°.

$$\text{C}_{30}\text{H}_{34}\text{O}_7. \text{ Calculated. C 71.09, H 6.77.}$$

$$\text{Found. " 70.73, " 6.97.}$$

<sup>7</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxxv, 501, 503.

## STROPHANTHIN.

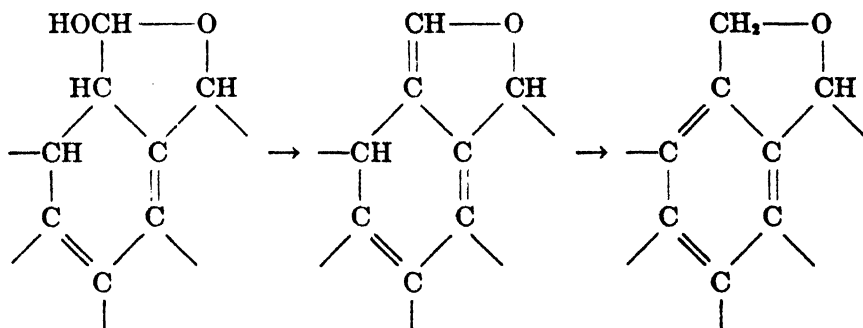
### XII. THE OXIDATION OF TRIANHYDROSTROPHANTHIDIN.

By WALTER A. JACOBS AND EDWIN L. GUSTUS.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, July 2, 1927.)

The assumption has been made, as discussed in the preceding paper, that the loss of water which occurs during the conversion of the oxidic form of dianhydrostrophanthidin into trianhydrostrophanthidin gives rise to a new double bond, which then shifts



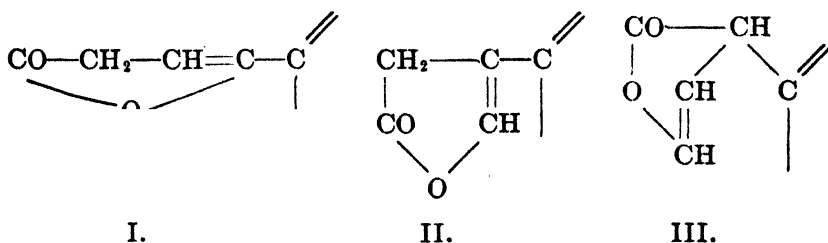
into the ring with the formation of a benzenoid structure. The failure of this substance to be hydrogenated with palladium except on the original  $\beta,\gamma$ -lactone double bond or to add bromine, in contrast with the behavior shown by dianhydrostrophanthidin, may be considered as good reasons for such a conclusion.

It was believed that additional evidence of such a structure might be obtained by studying the behavior of this substance towards oxidizing agents and that this procedure might offer also a promising method of approach to the oxidative degradation of the strophanthidin molecule. In all previous work successful oxidation experiments with strophanthidin or its derivatives have resulted in the isolation of substances in which the rings of the parent substance have remained intact, as will be definitely shown in the following communication.



The oxidation of trianhydrostrophanthidin, however, in agreement with the view that one of its rings is benzenoid in character, has given substances in which either degradation or ring cleavage has occurred. When oxidized in acetone solution with potassium permanganate, a crystalline acid was obtained, although in poor yield, while a good proportion of unchanged trianhydrostrophanthidin could be recovered. Analysis of this acid gave figures which were in best agreement with the formula  $C_{20}H_{24}O_3$ . This formulation was confirmed by the titration of the acid which indicated a monobasic acid of molecular weight 312; the calculated value is 312.2. It appeared therefore that the  $\Delta^{\beta,\gamma}$ -lactone group was the point of oxidation and was replaced by a carboxyl group with the loss of  $C_3H_2$ . This was directly confirmed by the failure of the new acid to consume more alkali on boiling with normal sodium hydroxide and by the fact that it no longer gave the nitroprusside test. Like the parent substance, it did not absorb bromine, indicating the retention of the benzenoid structure.

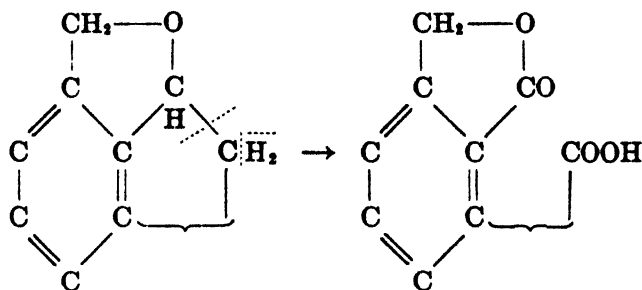
For the production of this substance by oxidation of the lactone group, two structural possibilities may be considered. Either the  $\Delta^{\beta,\gamma}$ -lactone is a straight chained lactone of a keto acid (I), or it is



a lactone of an aldehydo acid in which the  $\beta$  or  $\gamma$  carbon atom is the point of union with the benzenoid ring. The latter possibility, and particularly formula (II), has been chosen for reasons which will be presented in the following communication on isostrophanthidin.

As previously described, when dianhydrostrophanthidin was oxidized in acetone solution with permanganate, the only tangible crystalline product was the lactone acid  $C_{23}H_{28}O_6$  in which the free aldehyde group was changed to carboxyl and no suspicion of the above type of degradation product was obtained. More recently, all attempts to oxidize in a similar manner the dianhydrodilactone  $C_{23}H_{26}O_4$  resulted in failure. These substances, of course, cannot have a benzenoid ring.

Trianhydrostrophanthidin was more readily oxidized in acetic acid solution by chromic acid. The reaction mixture consisted largely of non-crystallizable neutral material from which about 30 per cent of a crystalline acid was isolated. The analysis of this acid gave figures which agreed with the formula  $C_{23}H_{24}O_6$ . This was confirmed by analysis of its ester. The acid was accordingly formed from trianhydrostrophanthidin by the addition of 3 oxygen atoms with the simultaneous loss of 2 hydrogen atoms. Direct titration showed the presence of one carboxyl group, but on boiling with alkali two additional equivalents were consumed. The substance still gave the nitroprusside test showing the retention of the  $\Delta^{\beta,\gamma}$ -lactone group. The additional equivalent of alkali was accounted for by the formation of a new lactone group. Finally the benzenoid structure appeared to be retained, since the substance did not absorb bromine. These facts may be satisfactorily explained by assuming, as illustrated in the accompanying formula,



that the carbon atom of the adjoining ring which carries the secondary hydroxyl  $OH^{III}$  of strophanthidin and which in trianhydrostrophanthidin is in oxidic union with the former aldehydic carbon is also attached to a  $CH_2$  group in the second ring. The bond between these carbon atoms is the point where oxidation occurs with rupture of the ring. The oxidic carbon becomes a lactone carbon and the  $CH_2$  is oxidized to carboxyl. This represents the first instance of successful ring cleavage with these substances, although more recently other observations have been made which will be incorporated in a subsequent communication.

When oxidized with chromic acid under the same conditions, dianhydrostrophanthidin, as previously reported, yields a dilactone but the carbon atom bearing  $OH^{III}$  is not affected. In the cases described in the previous paper, wherever  $OH^{III}$  is free it may

be oxidized to carbonyl by this reagent but the oxidation does not proceed beyond this point. Accordingly, the different behavior of this carbon atom in trianhydrostrophanthidin is in agreement with the assumption that it is now directly attached to a benzenoid ring.

#### EXPERIMENTAL.

*The Acid, C<sub>20</sub>H<sub>24</sub>O<sub>3</sub>.*—A solution of 4 gm. of trianhydrostrophanthidin in 100 cc. of carefully dried acetone was turbined with 4 gm. of dry powdered potassium permanganate. This was performed in a flask provided with a calcium chloride tube and a stirrer which was fitted with a mercury seal in order to exclude moisture during the reaction. The flask was kept chilled with ice. After 3 hours of turbining, the reagent was used up. The collected MnO<sub>2</sub> precipitate after washing with dry acetone was dried and then shaken thoroughly with 300 cc. of water. The filtrate on gentle acidification with hydrochloric acid gave an amorphous precipitate and a distinct odor resembling that of butyric acid. After standing, the collected and dried precipitate was recrystallized by careful dilution of its solution in a minimal volume of hot acetone. The yield was 0.35 gm. Recrystallized again from alcohol, it formed leaflets which melted at 206–208°. The substance in dilute pyridine solution does not give the Legal reaction with sodium nitroprusside.

0.1089 gm. of substance when titrated directly against phenolphthalein required 3.49 cc. of 0.1 N NaOH. For C<sub>20</sub>H<sub>24</sub>O<sub>3</sub>, the molecular weight is 312.2. Found, 312.

0.1046 gm. of substance required 3.31 cc. of 0.1 N NaOH. Found, molecular weight 316. When boiled with alkali, no evidence of the presence of a lactone group could be obtained.

0.1030 gm. was refluxed for 3 hours in 5.45 cc. of N NaOH and then titrated back. Found, 0.29 cc. of N NaOH. Calculated for 1 equivalent, 0.33 cc. When titrated with bromine by Winkler's method for double bonds, the absorption of bromine was negligible.

C <sub>20</sub> H <sub>24</sub> O <sub>3</sub> . Calculated.	C 76.87, H 7.75.
Preparation 1. Found. (a).	" 76.71, " 7.77.
	(b). " 76.71, " 7.64.
Preparation 2. Found.	" 76.53, " 7.52.

When the above washed MnO<sub>2</sub> residue was dissolved in bisulfite solution, a relatively small additional amount of this acid

was recovered after long standing. When the acetone solution obtained after filtering the above reaction mixture was concentrated, about 50 per cent of unchanged trianhydrostrophanthidin was recovered. When this material was again submitted to the above treatment with permanganate, additional amounts of the acid  $C_{20}H_{24}O_8$  were obtained, showing that this acid is unquestionably an oxidation product of trianhydrostrophanthidin itself and not of some difficultly detectable substance which might contaminate the former.

*The Acid,  $C_{23}H_{24}O_6$ .*—In the oxidation of trianhydrostrophanthidin with chromic acid, the yield of this crystalline acid was considerably affected by the amount of sulfuric acid employed. The best results after a number of experiments were obtained by using the following conditions.

A solution of 5 gm. of trianhydrostrophanthidin in 75 cc. of acetic acid was treated with a solution of 4 gm. of chromic acid in 20 cc. of 25 per cent sulfuric acid. The reaction which immediately started was controlled by keeping the temperature of the mixture below  $30^\circ$ . After 4 hours standing, the diluted mixture was extracted with chloroform. The chloroform extract was repeatedly washed with water to remove the excess of acetic acid and was finally extracted with dilute ammonia. After removal of dissolved  $CHCl_3$  from the ammoniacal extract, the latter was acidified with acetic acid, causing the immediate precipitation of the gradually crystallizing acid oxidation product. When recrystallized from dilute acetone, it formed slender prisms which melted at  $236-237^\circ$ . The yield was 1.4 gm. The substance gives the Legal reaction with sodium nitroprusside in dilute pyridine solution.

0.1000 gm. of substance dissolved in 5 cc. of alcohol was titrated directly against phenolphthalein with 0.1 N NaOH. Found, 2.48 cc. Calculated for 1 equivalent for  $C_{23}H_{24}O_6$ , 2.52 cc. An excess of 0.1 N NaOH was then added; the mixture was refluxed for 4 hours and then titrated back. An additional 4.31 cc. of 0.1 N NaOH were consumed. For 2 equivalents, 5.04 cc. are required.

0.1087 gm. of substance was refluxed for 2 hours in 2.18 cc. of N NaOH and titrated back. Found, 0.78 cc. of N NaOH. Calculated for 3 equivalents, 0.82 cc.

By the Winkler method of titration for double bonds, no ab-

sorption of bromine was noted. Attempts to benzoylate the acid or to prepare from it an oxime resulted in recovery of unchanged material.

$C_{23}H_{24}O_6$ .	Calculated.	C 69.66, H 6.11.
Preparation 1.	Found.	" 69.58, " 6.33.
" 2.	"	" 69.31, " 6.28.
" 3.	"	" 69.45, " 5.99.

The above chloroform solution which remained after extraction with dilute ammonia gave when concentrated a resinous mass from which no crystalline substance could be isolated.

*Methyl Ester of the Acid,  $C_{23}H_{24}O_6$ .*—The ester was prepared in acetone solution with diazomethane. After removing the solvent, the oily residue readily crystallized under alcohol. Recrystallized from methyl alcohol, it formed small prismatic needles which melted at 144–146° after slight preliminary softening.

$C_{24}H_{26}O_6$ .	Calculated.	C 70.21, H 6.39.
Preparation 1.	Found. (a).	" 70.31, " 6.46.
	(b).	" 70.36, " 6.36.
Preparation 2.	Found.	" 70.36, " 6.38.

## STROPHANTHIN.

### XIII. ISOSTROPHANTHIDIN AND ITS DERIVATIVES.

By WALTER A. JACOBS AND EDWIN L. GUSTUS.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, July 2, 1927.)

Strophanthidin in methyl alcoholic alkali is isomerized to  $\alpha$ -isostrophanthidin.<sup>1</sup> This was shown to involve the double bond, since such an isomerization could not be accomplished with dihydrostrophanthidin. Likewise, all attempts to hydrogenate isostrophanthidin with palladium and hydrogen were unsuccessful, and finally, this substance does not give the nitroprusside reaction characteristic of the  $\Delta^{\beta,\gamma}$ -lactone group. The isomerization, therefore, was due either to a simple shift in the double bond to a position where it lost its reactivity, or a new ring was formed with the disappearance of the double bond. In the work which will be here described, the latter possibility has been demonstrated.

As previously reported, isostrophanthidin is more resistant than strophanthidin to the action of permanganate in neutral acetone solution. After saponification in aqueous solution, it is readily oxidized by this reagent with the addition of 2 atoms of oxygen to  $\alpha$ -isostrophanthic acid,  $C_{23}H_{32}O_8$ , a saturated dibasic lactone acid. With alkaline hypobromite, isostrophanthidin is oxidized by the addition of 1 atom of oxygen to  $\alpha$ -isostrophanthidic acid,  $C_{23}H_{32}O_7$ , a monobasic aldehydolactone acid which by further oxidation of the aldehyde group to carboxyl can be converted into  $\alpha$ -isostrophanthic acid. In these substances we had assumed the probable identity of their lactone groups with those of isostrophanthidin and strophanthidin. On continuing the study of the substances, this view has been found to be incorrect.

<sup>1</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lxi, 387.

More recent investigations have shown that isostrophanthidin as such is not oxidized by neutral hypobromite, even in dilute pyridine solution, and, similarly, with neutral permanganate, isostrophanthic acid cannot be obtained. Preliminary saponification has been found to be an essential prerequisite. It appeared, therefore, that the point attacked by the reagents to form the new carboxyl group is a group liberated by saponification. Since the formulation and properties of both isostrophanthidic acid and isostrophanthic acid do not fit with the assumption that ring cleavage has occurred during their formation, it appeared that the liberated and subsequently oxidized group was an aldehyde group which would require but 1 atom of oxygen for conversion to carboxyl.

The attempt to determine this directly by the preparation of a dioxime from saponified isostrophanthidin led to no tangible result. It was difficult to ascertain whether the desired reaction had occurred owing to the solubility of the reaction product, both as salt and free acid. Acidification to Congo red gave only the oxime of isostrophanthidin.<sup>2</sup> The evidence sought was obtained, however, as follows, from the ester of strophanthidinic acid,<sup>3</sup> in which the complicating reactive free aldehyde group was disposed of.

Contrary to strophanthidinic acid itself, its methyl ester was found to behave like strophanthidin when dissolved in methyl alcoholic alkali and isomerized to a beautifully crystallizing and sparingly soluble *α-isostrophanthidinic methyl ester*. That the isomerization here paralleled that observed in the formation of isostrophanthidin was shown as follows. The lactone group of this iso ester, just as in the case of strophanthidinic methyl ester, was much more readily saponified than its ester group with the formation of *α-isostrophanthidindiacid monomethyl ester*.

Although the unsaponified isolactone ester, like isostrophanthidin, was not affected by neutral hypobromite in dilute pyridine

<sup>2</sup> In view of what follows, the desired dioxime was probably formed; but the hydrolyzing action at this pH, supported by the tendency to relactonize, was probably responsible for the isolation of only a neutral monoxime.

<sup>3</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1927, lxxiv, 796.

solution, when the lactone group was once opened the resulting acid as the neutral salt was readily oxidized to a substance which was found to be the monomethyl ester of a dibasic lactone acid. This proved to be identical with  $\alpha$ -isostrophanthic monomethyl ester, which could also be obtained by the partial saponification of  $\alpha$ -isostrophanthic dimethyl ester. The nature and origin of the lactone group of this oxidation product will be discussed below.

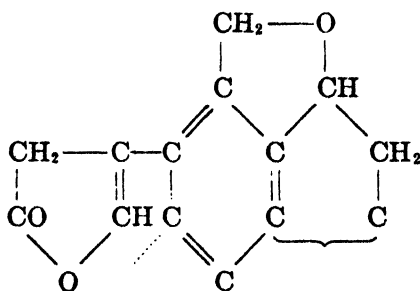
The above  $\alpha$ -isostrophanthidindiacid monomethyl ester was found to react as the salt with both hydroxylamine and semicarbazide, but the resulting substances did not lend themselves to ready manipulation. Better success was obtained with the neutral dimethyl ester which yielded a beautifully crystalline semicarbazone. The group liberated, therefore, on saponification of the original lactone group is of aldehydic character which is oxidized by 1 atom of oxygen to carboxyl followed by subsequent relactonization.

The next question of importance was the identification of the carboxyl and hydroxyl groups involved in the new lactone formation. This new lactone group appeared to be  $\gamma$  or  $\delta$  in character, because of the ease with which its saponification and relactonization could be accomplished. This question was satisfactorily answered in the following way.  $\alpha$ -Isostrophanthic dimethyl ester,  $C_{25}H_{36}O_8$ , was found to be readily oxidized with chromic acid to a ketone,  $\alpha$ -isostrophanthonic dimethyl ester,  $C_{25}H_{34}O_8$ , which yielded an oxime but no longer gave a benzoate. The ketone now exhibited a great lability, since its remaining hydroxyl was readily removed by acid with the formation of an unsaturated ketone, anhydro- $\alpha$ -isostrophanthonic dimethyl ester,  $C_{25}H_{32}O_7$ , which could be readily hydrogenated to two isomeric dihydro derivatives. The formation of all these substances paralleled exactly that of those obtained by the oxidation of strophanthidinic methyl ester previously reported. Therefore, in this series of substances, the secondary hydroxyl,  $OH^{III}$ , is first oxidized to the ketone, and subsequently  $OH^{II}$  is removed as water. In isostrophanthic dimethyl ester,  $OH^{II}$  and  $OH^{III}$  must, therefore, be retained, which leaves  $OH^I$  as the group involved in the formation of the new lactone ring. In a sense this is compatible with the fact shown in a previous communication, that strophanthidinic



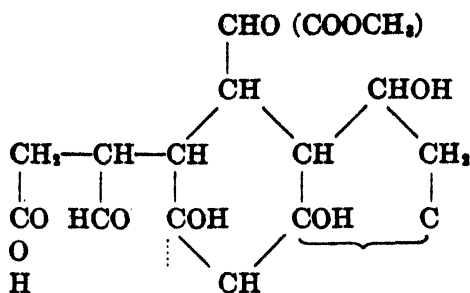
lactone<sup>4</sup> is the internal ester on the same hydroxyl group ( $\text{OH}^1$ ), but, however, of a different carboxyl group. It is curious that  $\text{OH}^1$ , which cannot be directly acylated by the usual methods, can function here in two types of internal esters.

In order to determine whether the carboxyl group which formed the lactone ring in  $\alpha$ -isostrophanthic acid is that formerly involved in the lactone ring of strophanthidin and isostrophanthidin itself or is the one newly formed from the liberated aldehyde group, the attempt was made to oxidize  $\alpha$ -isostrophanthidindimethyl ester, as in the case of the monomethyl ester, with neutral hypobromite. Strangely enough, no reaction occurred. However, chromic acid oxidized this substance at once, but carried it, of course, a step farther, to the previously mentioned  $\alpha$ -isostrophanthonic dimethyl ester. This was confirmed by its dehydration to the unsaturated anhydro- $\alpha$ -isostrophanthonic dimethyl ester. In both isostrophanthidic and isostrophanthic acids, therefore, the lactone group involves  $\text{OH}^1$  and the carboxyl group which arises from the aldehyde group which is liberated when isostrophanthidin is saponified. Since in the former communication oxidation experiments with trianhydrostrophanthidin have shown the original lactone group to be a side chain, the present work completes this by demonstrating that this lactone ring is the  $\Delta^{\beta,\gamma}$ -lactone of an enolized aldehydo acid (I). Similarly, the carbon of this aldehyde group is either  $\gamma$  or  $\delta$  to  $\text{OH}^1$ . Placing it in the  $\gamma$  position, as shown in the accompanying graphic formula, is more in harmony with the observed facts.

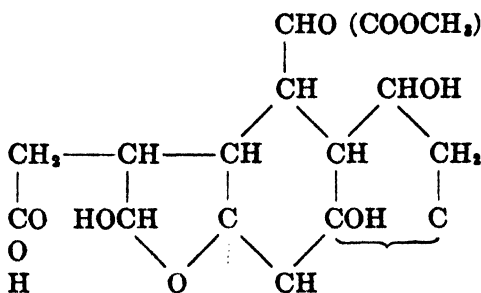


1.

<sup>4</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1927, lxxiv, 798.



## II.

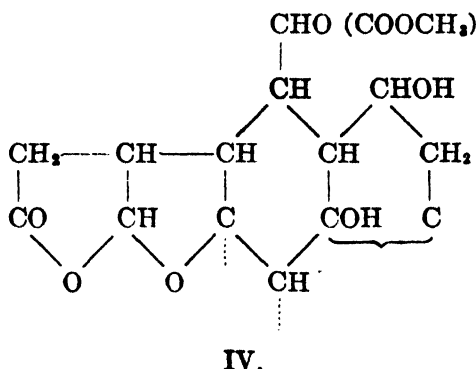


### III.

Accordingly, when isostrophanthidin and isostrophanthidinic methyl ester are saponified, their lactone groups open with the formation of a hydroxyaldehyde, which may exist as the free aldehyde (II), or possibly in the oxidic form (III). The latter possibility was suggested by the fact that when neutral hypobromite was used for oxidation of saponified isostrophanthidin or isostrophanthidindiacid monomethyl ester, the lactone group appeared to be directly formed, since gentle acidification of the reaction mixture caused a prompt crystallization of the lactone compound. When the lactone group is actually saponified, considerable time is required for relactonization under such conditions. This suggestion was directly borne out in an attempt to prepare the dimethyl ester of  $\alpha$ -isostrophanthidindiacid with methyl iodide and excess alkali. Instead of the dimethyl derivative obtained with diazomethane which, as described before, yielded a semicarbazone, here a trimethyl derivative was obtained in which the additional methyl was bound on the oxidic form of the aldehyde as an oxidohalfacetal. This substance no

longer formed a semicarbazone and when hydrolyzed with acid yielded, with simultaneous saponification of the labile ester group and relactonization,  $\alpha$ -isostrophanthidinic methyl ester.

The fact that the hydroxyaldehyde can exist in the oxidic form suggested the possibility that the failure to detect a double bond in isostrophanthidin was due to the fact that it had disappeared during the formation of this substance. Instead, a new oxidic ring has been formed, and this substance has become the inner ester of the oxidic form of the aldehyde, as is more readily seen from the accompanying graphic representation (IV).



In accordance with this, isostrophanthidin and isostrophanthidinic methyl ester should possess only two free hydroxyl groups. This was directly demonstrated by determining the active hydrogen atoms by the Zerewitinoff method. For comparison, strophanthidin itself was not used because of its water content. Instead, anhydrous dihydrostrophanthidin which no longer possesses the lactone active hydrogen was employed. It showed the required 3 active hydrogen atoms. Similarly, strophanthidinic methyl ester, which has in addition the lactone active hydrogen, showed 4 active hydrogen atoms. On the other hand, isostrophanthidin and the analogous iso ester both showed only 2. The transformation of strophanthidin and strophanthidinic methyl ester into iso compounds is, therefore, accompanied by the disappearance of the double bond with the formation of a new oxidic ring on OH<sup>1</sup>. The exact mechanism of this rearrangement is not entirely clear, but it is probably induced by the enolization of the carbonyl of the aldehyde or ester group with the formation of a double bond, since substances of the series which do not

possess this carbonyl group, such as strophanthidin and pseudostrophanthidin, do not seem to undergo this rearrangement.

This may be regarded as additional evidence of the presence of a CH group adjoining the aldehyde group, a fact already indicated by the conversion of dianhydrostrophanthidin into trianhydrostrophanthidin. The oxidation products thus far obtained in the isostrophanthidin series still contain intact the four saturated rings of the parent substance, strophanthidin, and with a proper interpretation of their relationship to this substance it is now possible to use them more intelligently in further studies.

It is highly interesting that the reactive groups of strophanthidin are so closely associated in the same general portion of the molecule. This has made possible the ready formation of at least four different types of oxidic or lactone rings, which have been encountered up to the present in our studies and have given rise to much confusion before their identities were determined.

#### EXPERIMENTAL.

*Preparation of Isostrophanthidin.*—Later experience with the method previously given<sup>5</sup> in which the alkaline solution of strophanthidin was allowed to stand at room temperature was not entirely satisfactory. This was due to secondary reactions which resulted occasionally in the production of various resinous by-products which greatly diminished the yield of isostrophanthidin. The following modification of the method was found to give reliable results where the temperature was controlled. 50 gm. of strophanthidin were dissolved in a solution of 12 gm. of potassium hydroxide in 500 cc. of methyl alcohol. The solution was kept at 5–8° for 6 hours, or until the test with sodium nitroprusside had practically disappeared. The solution was then gently acidified by the addition of 25 cc. of 50 per cent acetic acid and diluted with 4 volumes of cold water. The separation of the unsaponified isostrophanthidin was completed by removal of the methyl alcohol at low pressure. 27 gm. were obtained directly. The mother liquor was then acidified to Congo red with hydrochloric acid. On standing, relactonization with the gradual

<sup>5</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lxi, 391.

crystallization of additional substance occurred, which amounted to 15 gm. After recrystallization from methyl alcohol, the substances separated as anhydrous glistening leaflets in each case and except for a constant slight difference in melting point were identical in all respects. The unsaponified  $\alpha$ -isostrophanthidin melted at 259–261°, while the substance recovered after relactonization melted at 274–276°. The former substance gave  $[\alpha]_D^{28} = +48^\circ$  ( $c = 6.667$  in pyridine); the latter gave  $[\alpha]_D^{28} = +49.3^\circ$  ( $c = 6.667$  in pyridine). Likewise, as presented later, each substance gave 2 mols of methane with Grignard reagent. There appears to be no question of their identity, since the transformation products were also identical in each case.

*$\alpha$ -Isostrophanthic Monomethyl Ester.*—The greater stability of one of the ester groups of the dimethyl ester towards alkali was shown by the following titration experiment. 0.1000 gm. of  $\alpha$ -isostrophanthic dimethyl ester<sup>6</sup> was refluxed for 2 hours in a mixture of 10 cc. of alcohol and 10 cc. of 0.1 N NaOH, and the solution was then titrated against phenolphthalein. Calculated for 2 equivalents, 4.30 cc. Found, 3.77 cc., a portion of which was used for saponification of the lactone group. For preparative purposes, the following method was used.

The dimethyl ester was shaken at 20° in a mixture of 20 parts of 2 per cent sodium hydroxide solution and 20 parts of alcohol. After 30 minutes, solution was complete. When acidified with hydrochloric acid, the half ester easily crystallized. From methyl alcohol, it formed broad needles which melted at 235–237°, although occasionally a melting point of 214° was observed. It is readily soluble in the alcohols, acetone, and chloroform.

$$[\alpha]_D^{28} = +13^\circ \text{ (} c = 1.117 \text{ in pyridine).}$$

*Air-Dry Substance.* Dried at 100° and 15 mm. over  $H_2SO_4$ .

$C_{24}H_{34}O_8 \cdot H_2O$ . Calculated.  $H_2O$  3.85.

Found. " 4.02.

*Anhydrous Substance.*

$C_{24}H_{34}O_8$ . Calculated. C 63.96, H 7.61.

Found. " 63.74, " 7.62.

<sup>6</sup> The dimethyl ester employed in these experiments has been prepared in larger amounts more conveniently than by the diazomethane method from the disodium salt in absolute methyl alcohol with methyl iodide.

*Oxidation of  $\alpha$ -Isostrophanthic Dimethyl Ester.*

*$\alpha$ -Isostrophanthonic Dimethyl Ester.*—A solution of  $\alpha$ -isostrophanthic dimethyl ester<sup>6</sup> in 10 parts of acetic acid was treated with a slight excess of Kiliani chromic acid solution. Oxidation occurred rapidly. After 15 minutes, dilution of the mixture caused a rapid separation of the keto ester in excellent yield. It formed from methyl alcohol sparingly soluble, small, stout needles or plates, often in arborescent masses, and melted, but not sharply, at 254–255°. It is most easily soluble in chloroform and pyridine, less readily so in acetone, and but sparingly soluble in the alcohols.

$$[\alpha]_D = -6.2^\circ \text{ (c = 1.13 in pyridine).}$$

$C_{25}H_{34}O_8$ .	Calculated.	C 64.90,	H 7.41.
	Found.	" 64.62,	" 7.44.

Attempts to benzoylate the substance resulted only in recovery of unchanged material.

*$\alpha$ -Isostrophanthonic Dimethyl Ester Oxime.*—The oxime was prepared in methyl alcoholic solution with hydroxylamine hydrochloride and sodium acetate. It formed delicate needles from dilute methyl alcohol which melted with effervescence at 228°.

$$C_{25}H_{36}O_8N.$$

	Calculated.	C 62.86,	H 7.39.
	Found.	" 62.61,	" 7.13.

*Anhydro- $\alpha$ -Isostrophanthonic Dimethyl Ester.*—For conversion into the anhydro compound, the crude keto ester obtained from 19.5 gm. of  $\alpha$ -isostrophanthic dimethyl ester was employed without previous recrystallization. This was suspended in 300 cc. of alcohol and 6 cc. of hydrochloric acid (1.19) and refluxed for 30 minutes. Solution occurred rapidly. On cooling, the anhydro compound crystallized in excellent yield. From methyl alcohol, it separated as minute, stout wedges or rhombs which did not melt sharply. It melted at 210° after considerable preliminary softening. In all other ways the substance appeared to be homogeneous. Longer heating of the original reaction mixture did not affect the result.

$$[\alpha]_D^{25} = +74^\circ \text{ (c = 1.023 in pyridine).}$$

The resistant ester group is retained in this substance, as shown by titration. 0.1005 gm. of substance was boiled for 2 hours in a

mixture of 10 cc. of alcohol and 10 cc. of 0.1 N NaOH and titrated against phenolphthalein. Calculated for 2 equivalents, 4.52 cc. Found, 4.57 cc.

$C_{25}H_{32}O_7$ . Calculated. C 67.53, H 7.26.  
Found. " 67.62, " 7.41.

*Anhydro- $\alpha$ -Isostrophanthonic Monomethyl Ester*.—1 gm. of the dimethyl ester was warmed for a few minutes in 20 cc. of 2 per cent sodium hydroxide solution and 20 cc. of methyl alcohol until dissolved. When diluted and acidified with acetic acid, it crystallized. From methyl alcohol, it separated as serrated, wedge-shaped crystals which did not show a real melting point. It slowly softened above 245° to a resin which finally melted at 260°.

$C_{24}H_{30}O_7$ . Calculated. C 66.94, H 7.03.  
Found. " 66.87, " 6.94.

*Desoxy- $\alpha$ -Isostrophanthonic Dimethyl Ester*.—4 gm. of anhydro- $\alpha$ -isostrophanthonic dimethyl ester were hydrogenated in acetic acid solution with 0.4 gm. of palladium black. The absorption of 1 mol of hydrogen occurred during the 1st hour and then practically ceased. Dilution of the filtrate caused separation of a mixture of two substances, platelets and stout prisms. Separation was accomplished by taking advantage of the greater solubility of the platelets in acetone. The mixture was obtained in several fractions from methyl alcohol. On digestion of each fraction with acetone, the more sparingly soluble substance remained as a sandy powder which was finally recrystallized from acetone. It formed stout, rectangular prisms which softened first to a paste at 200° but were not completely melted until 206° was reached. The melting point and rotation were not changed by repeated recrystallization.

$[\alpha]_D = -4^\circ$  (c = 0.7 in pyridine).  
 $C_{26}H_{34}O_7$ . Calculated. C 67.22, H 7.68.  
Found. " 67.19, " 7.62.

The more soluble isomer was obtained from the acetone washings and mother liquors. Recrystallized from methyl alcohol, it

formed leaflets which melted at 207–208° after preliminary softening.

$$[\alpha]_D = -57^\circ \text{ (c = 0.7 in pyridine).}$$

$C_{21}H_{34}O_7$ . Calculated. C 67.22, H 7.68.  
Found. " 67.47, " 7.80.

*Derivatives of  $\alpha$ -Isostrophanthidinic Acid.*

*$\alpha$ -Isostrophanthidinic Methyl Ester.*—3 gm. of strophanthidinic<sup>3</sup> methyl ester were dissolved in 30 cc. of a methyl alcoholic solution of potassium hydroxide containing 2.4 gm. per 100 cc. The solution was left at 20° for 6 hours, when the nitroprusside test had become negative. Dilution with water caused the crystallization of a small amount of neutral substance which proved to be isomerized ester. The majority remained in solution due to saponification of the lactone group. On acidification with acid to Congo red, the remainder gradually separated due to relactonization. When recrystallized from absolute methyl alcohol, needles were obtained which melted at 270–271° with effervescence; although the melting point appeared to be greatly affected by the conditions of recrystallization, since unquestionably pure samples were obtained which melted considerably below this point (242°, 250–252°, 257°), but which on long standing in the dry state were transformed into the high melting form. The substance is appreciably soluble in chloroform and in acetone, and sparingly soluble in the alcohols. It does not give a positive nitroprusside reaction.

$$[\alpha]_D^{25} = +84^\circ \text{ (c = 1.003 in pyridine).}$$

$C_{21}H_{34}O_7$ . Calculated. C 66.33, H 7.89.  
Found. " 66.12, " 7.86.

*$\alpha$ -Isostrophanthidindiacid Monomethyl Ester.*—5 gm. of strophanthidinic methyl ester were isomerized as above and then diluted with water. The alcohol was removed carefully at room temperature without allowing the mixture to become too concentrated. The acid was obtained on acidification with acetic acid as aggregates of broad rods. When recrystallized from methyl alcohol, it separated in the same form and melted at 204–206°. The yield was 3.4 gm. The substance was completely



soluble in dilute ammonia, but was readily lactonized by mineral acid. As a matter of fact, this occurred slowly even with a weak acid, such as acetic acid, since the mother liquor of the freshly precipitated acid slowly deposited a second fraction of neutral lactone which was formed from that portion of the acid which had remained in solution.

$C_{24}H_{36}O_8$ . Calculated. C 63.67, H 8.02.  
Found. " 63.61, " 7.97.

This acid is a hydroxyaldehyde which can exist either in the aldehydic or oxidic form, as shown by its behavior.

When a solution of the sodium salt of this acid was heated with hydroxylamine or semicarbazide under neutral conditions, a reaction occurred. However, only in the case of the oxime was it possible to isolate a crystalline substance. This was obtained after acidification of the reaction mixture with acetic acid by concentration at room temperature to small volume, which caused the separation of leaflets. This substance, which contained nitrogen and was unquestionably the desired oximino acid, proved to be very soluble, which rendered its isolation and purification difficult with the material available. We had recourse, therefore, rather to its ester, which was prepared with diazomethane. With methyl iodide, an additional methyl group was introduced, forming the methylhalfacetal, as given below.

*$\alpha$ -Isostrophanthidindiacid Dimethyl Ester.*—A suspension of the previous monomethyl ester in acetone rapidly dissolved when treated with diazomethane. The concentrated solution deposited the ester, which was again recrystallized from acetone. It formed rods and long prisms which were readily soluble in chloroform and in hot methyl or ethyl alcohol and acetone. It melted at 226–228°.

$C_{26}H_{38}O_8$ . Calculated. C 64.33, H 8.21,  $OCH_3$  13.30.  
Found. " 64.09, " 8.44, " 13.41.

From the mother liquor obtained by recrystallizing the ester, a small amount of a substance was recovered which melted at 270° and which analysis showed to be  $\alpha$ -isostrophanthidinic methyl ester. This was formed probably by partial relactonization of the acid during the preparation of the dimethyl ester.

The dimethyl ester itself showed no tendency to lactonize when boiled for 6 hours in neutral methyl alcohol, so that manipulation during recrystallization was not responsible for the presence of this high melting substance.

Although the dimethyl ester reacted with hydroxylamine, the resulting oxime showed no tendency to crystallize. The semicarbazone proved to be a substance which crystallized readily.

*$\alpha$ -Isostrophanthidindiacid Dimethyl Ester Semicarbazone.*—A solution of 1.6 gm. of the dimethyl ester in 125 cc. of alcohol was treated successively with 1.6 gm. of potassium acetate dissolved in 8 cc. of alcohol and 1.5 gm. of semicarbazide hydrochloride dissolved in 20 cc. of water. The precipitated potassium chloride was redissolved by subsequent addition of sufficient water. The solution was allowed to stand at 40° for 36 hours. When the alcohol was removed at low pressure, the clear solution deposited a thick mass of needles and long prisms. After several recrystallizations from methyl alcohol, it crystallized in the same form and melted at 214–215° to a mass of bubbles. The substance contained solvent of crystallization, which was very difficult to remove completely, even on drying at 110° at low pressure. As a result, the analytical figures obtained with this substance were not entirely satisfactory. This was corrected by recrystallizing the substance by the addition of water to its methyl alcoholic solution with subsequent removal of the alcohol at 25° under reduced pressure. The substance separated as slender needles which when air-dried melted at 144–146° to a mass of bubbles and contained water of crystallization.

*Air-Dry Substance.* Dried at 110° and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{26}\text{H}_{41}\text{O}_8\text{N}_3 \cdot 2\frac{1}{2}\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  7.92.

Found. " 8.15.

*Anhydrous Substance.*

$\text{C}_{26}\text{H}_{41}\text{O}_8\text{N}_3$ . Calculated. C 59.61, H 7.90, N 8.03,  $\text{OCH}_3$  11.86.

Found. " 59.79, " 8.17, " 8.01, " 11.88.

*Methylhalfacetal of Oxido- $\alpha$ -Isostrophanthidindiacid Dimethyl Ester.*—2.6 gm. of strophanthidinic methyl ester were isomerized as usual in methyl alcoholic potassium hydroxide solution, but the mixture was left at room temperature for 18 hours in order to insure complete saponification of the lactone group.

The solution was repeatedly concentrated at low pressure after addition of dry methyl alcohol in order to remove water. The volume, however, was not allowed to drop below 10 to 20 cc., in order to avoid saponification of the remaining ester group. Finally, in a volume of 50 cc., 6 cc. of methyl iodide were added and the mixture was refluxed for 9 hours. The concentrated solution readily crystallized on dilution. When recrystallized from acetone, it formed pointed prisms and stout tablets which melted, not sharply, at 214–216°. When recrystallized from methyl alcohol, wedge-shaped and pointed prisms were obtained which melted at 219–220°. Although it resembled closely in properties the aldehyde dimethyl ester, analysis showed it to be a trimethyl derivative.

$C_{26}H_{40}O_8$ .	Calculated.	C 64.97,	H 8.40,	OCH <sub>3</sub> 19.37.
	Found.	" 64.73,	" 8.71,	" 19.16.

Contrary to the aldehyde ester, it was recovered unchanged in an attempt to react it with hydroxylamine. When boiled in methyl alcoholic solution to which aqueous hydrochloric acid had been added, the methyl acetal group was gradually hydrolyzed and simultaneously relactonization of the methyl ester group occurred with the formation of  $\alpha$ -isostrophanthidinic methyl ester.

#### *Oxidation of $\alpha$ -Isostrophanthidindiacid Esters.*

*Oxidation of  $\alpha$ -Isostrophanthidindiacid Monomethyl Ester.*—In attempts to oxidize  $\alpha$ -isostrophanthidinic methyl ester in dilute pyridine solution with neutral hypobromite, it remained unaffected. When the lactone group, however, was once saponified, the liberated aldehyde group in the oxidic form was at once oxidized by the reagent, even in neutral solution, with the formation of  $\alpha$ -isostrophanthic monomethyl ester.

To a solution of 0.5 gm. of the lactone methyl ester in 25 cc. of pyridine, 9 cc. of 0.5 N sodium hydroxide were added. The precipitated substance redissolved within a few minutes on shaking, due to saponification of the lactone group only. The mixture was carefully neutralized to phenolphthalein with hydrobromic acid, and was then treated with a carefully neutralized aqueous solution of hypobromite made from 0.3 gm. of bromine. After 1

hour, the mixture was acidified to Congo red. The acid crystallized at once. After further standing, the collected substance was recrystallized first from dilute methyl alcohol, forming long, flat needles or platelets which melted at 215–217°. When recrystallized from methyl alcohol, it melted at 232–234°, thus exhibiting the variable melting point of the previously described  $\alpha$ -isostrophanthic monomethyl ester. In sulfuric acid, it gradually developed the characteristic purple-red color of isostrophanthic acid and its esters.

$$[\alpha]_D^{25} = +14^\circ \text{ (c = 1.083 in pyridine).}$$

0.0826 gm. of anhydrous substance was dissolved in 3 cc. of alcohol, and on direct titration against phenolphthalein required 0.20 cc. of N sodium hydroxide solution. Calculated for 1 equivalent, 0.185 cc. 3 cc. of N alkali were then added, and the solution was boiled for 2 hours and again titrated back. Found, 0.34 cc. Calculated for 2 equivalents (lactone and ester groups), 0.37 cc.

*Air-Dry Substance.* Dried at 100° and 15 mm. over  $H_2SO_4$ .

$C_{24}H_{34}O_8 \cdot H_2O$ . Calculated.  $H_2O$  3.85.

Found. " 4.02.

*Anhydrous Substance.*

$C_{24}H_{34}O_8$ . Calculated. C 63.96, H 7.61.

Found. " 63.56, " 7.95.

*Oxidation of  $\alpha$ -Isostrophanthidindiacid Dimethyl Ester.*—In order to establish definitely the identity of the carboxyl group involved in the lactone grouping of isostrophanthic acid, an attempt was made to oxidize the diacid dimethyl ester with hypobromite under the conditions used above for the monomethyl ester. Strangely enough, no reaction occurred and unchanged material was recovered. Chromic acid, however, gave the desired result. In this case the dimethyl ester of  $\alpha$ -isostrophanthonic acid was formed due to simultaneous oxidation of the secondary hydroxyl to the ketone. The identification of this was confirmed by conversion into the anhydro keto ester.

2 gm. of the dimethyl ester were dissolved in 20 cc. of acetic acid and treated with 10 cc. of Kiliani solution. The immediate reaction which occurred was controlled by keeping the tempera-

ture below 30°. After 20 minutes, dilution caused the prompt separation of  $\alpha$ -isostrophanthonic dimethyl ester. After recrystallization from methyl alcohol, it melted at 250° and proved to be identical with the ketone obtained from isostrophanthic dimethyl ester as previously described.

$$[\alpha]_D^{25} = -9.4^\circ \text{ (c = 1.067 in pyridine).}$$

0.1004 gm. of substance was refluxed for several hours with 3 cc. of N sodium hydroxide solution and titrated against phenolphthalein. Calculated for 3 equivalents, 0.67 cc. Found, 0.65 cc.

$C_{25}H_{34}O_8$ .	Calculated.	C 64.90, H 7.41.
	Found.	" 64.86, " 7.65.

This was converted into the anhydro derivative as previously described. The usual stout wedges were obtained, which melted at 210–211°.

$$[\alpha]_D^{25} = +74^\circ \text{ (c = 1.023 in pyridine).}$$

$C_{25}H_{32}O_7$ .	Calculated.	C 67.53, H 7.26.
	Found.	" 66.99, " 7.57.

#### *Comparative Active Hydrogen Determinations.*

For the active hydrogen determinations, the method of Tschugaeff-Zerewitinoff was used. Only anhydrous substances were, of course, employed. After addition of the Grignard reagent, the mixture was shaken vigorously for 1 minute and then, after waiting 2 minutes for temperature equilibrium, the reading was taken.

- 0.0505 gm. dihydrostrophanthidin gave 8.7 cc.  $CH_4$  (25°, 764 mm.), or 2.82 mols for mol. wt. 406.
- 0.0506 gm. dihydrostrophanthidin gave 9.5 cc.  $CH_4$  (26°, 757 mm.), or 3.03 mols for mol. wt. 406.
- 0.0518 gm. strophanthidinic methyl ester gave 12.8 cc.  $CH_4$  (26°, 757 mm.), or 4.27 mols for mol. wt. 434.
- 0.0496 gm.  $\alpha$ -isostrophanthidin (A) gave 6.2 cc.  $CH_4$  (25°, 764 mm.), or 2.03 mols for mol. wt. 404.
- 0.0504 gm.  $\alpha$ -isostrophanthidin (A) gave 6.2 cc.  $CH_4$  (25°, 765 mm.), or 2.00 mols for mol. wt. 404.
- 0.0500 gm.  $\alpha$ -isostrophanthidin (B) gave 7.2 cc.  $CH_4$  (25°, 764 mm.), or 2.34 mols for mol. wt. 404.

0.0501 gm.  $\alpha$ -isostrophanthidin (B) gave 5.9 cc.  $\text{CH}_4$  ( $25^\circ$ , 765.4 mm.), or 1.92 mols for mol. wt. 404.

0.0510 gm.  $\alpha$ -isostrophanthidinic methyl ester gave 5.5 cc.  $\text{CH}_4$  ( $25^\circ$ , 765.4 mm.), or 1.89 mols for mol. wt. 434.

In the above tabulation, isostrophanthidin (A) was material which had been obtained from strophanthidin without saponification of the lactone group, and isostrophanthidin (B) was substance which had been obtained after saponification with subsequent relactonization. In their behavior towards Grignard reagent, however, no difference was noted.

Dihydrostrophanthidin, which has three hydroxyl groups, showed the 3 required active hydrogen atoms. Similarly, the expected result was obtained with strophanthidinic methyl ester, which possesses in addition the active hydrogen of the  $\alpha$ -carbon atom of the  $\Delta^{\beta,\gamma}$ -lactone group.

Since isostrophanthidin and  $\alpha$ -isostrophanthidinic methyl ester showed only 2 active hydrogen atoms, their formation, as discussed in the introduction, has involved the loss of the lactone active hydrogen atom and one hydroxyl group.



## STROPHANTHIN.

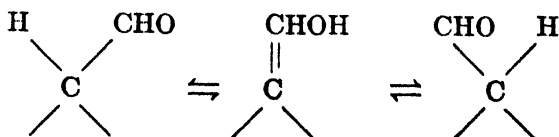
### XIV. ISOMERIZATION IN THE ISOSTROPHANTHIDIN SERIES.

BY WALTER A. JACOBS AND EDWIN L. GUSTUS.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

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In a previous communication<sup>1</sup> on the isomerization and oxidation of isostrophanthidin, two series of substances were described which were designated  $\alpha$  and  $\beta$ , respectively.  $\alpha$ -Isostrophanthidin is the form which was directly obtained when isomerization was performed at room temperature. But when  $\alpha$ -isostrophanthidin or its oxidation product  $\alpha$ -isostrophanthidic acid, which still contains the aldehyde group, was subjected to the action of hot dilute alkali, isomerization occurred with the formation of  $\beta$  compounds which were not isolated as such, but were detected by their conversion on oxidation into  $\beta$ -isostrophanthic acid. When the aldehyde group, however, was changed to carboxyl, this transformation could no longer be accomplished;  $\alpha$ -isostrophanthic acid could not be converted into  $\beta$ -isostrophanthic acid by boiling alkali. It appeared, therefore, that the isomerization was of the nature of a rearrangement of the aldehyde group and a hydrogen atom attached to the



adjoining asymmetric carbon atom, which temporarily lost its asymmetry due to enolization of the carbonyl group. As a matter of fact, with either  $\alpha$ -isostrophanthidin or  $\alpha$ -isostrophanthidic acid, the rearrangement seemed to reach an equilibrium, since the resulting substance was always a mixture of the  $\alpha$  and  $\beta$  compounds.

In more recent work, although attempts to isolate  $\beta$ -isostro-

<sup>1</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lxi, 387.



phanthidin from such a mixture have not met with success, it has been possible to accomplish this in the case of  $\beta$ -isostrophanthidic acid. This acid was found to yield a sparingly soluble ammonium salt which permitted its separation from the  $\alpha$  isomer. A study of the behavior of  $\beta$ -isostrophanthidic acid towards oxidizing agents has shown its exact relationship to the  $\alpha$  acid. Potassium permanganate was found to convert it quantitatively into  $\beta$ -isostrophanthic acid. On the other hand, hypobromite yielded a monobasic acid,  $C_{23}H_{30}O_7$ , which proved to be identical with the acid previously described as a by-product in the preparation of isostrophanthidic acid from isostrophanthidin<sup>2</sup> with the same reagent. From titration experiments, this acid had then been considered to be a monobasic lactone acid, but more recently a reconsideration of this conclusion has become necessary. Since this acid no longer yielded a benzoate or an oxime, it was evident that its formation must have involved both the acylatable secondary hydroxyl and the aldehyde group of  $\beta$ -isostrophanthidic acid. Since its derived formula differs from that of isostrophanthic acid,  $C_{23}H_{32}O_8$ , by 1 water molecule, the possibility appeared that a new but relatively resistant lactone group may have arisen during its formation which escaped detection by the method of titration previously employed. This was demonstrated by saponification with stronger alkali. On gentle reacidification, only one lactone group closed again, and the resulting dibasic lactone acid proved to be identical with  $\beta$ -isostrophanthic acid. The new lactone group of  $\beta$ -isostrophanthic lactone acid is, therefore, analogous to that encountered in the dilactone obtained by oxidation of dianhydrostrophanthidin. In both substances the carboxyl group forms an inner ester with the secondary hydroxyl ( $OH^{III}$ ) of the adjoining ring. This was confirmed by the fact that the above monobasic dilactone acid, contrary to isostrophanthic acid, is not affected by chromic acid under the usual conditions. The greater resistance of the second lactone group to saponification is but another example of the general stability in this respect of the esters of this particular carboxyl group, as discussed in the last two papers of this series.

<sup>2</sup> This by-product of the earlier experiments had its origin in  $\beta$ -isostrophanthidin, which contaminated the isostrophanthidin which was previously used. As we had already noted, this substance was not formed when pure  $\alpha$ -isostrophanthidin was used.

Since the resistant lactone group of the monobasic dilactone acid had been formed in an alkaline oxidizing medium, it must have arisen from the direct oxidation of the oxidic form of  $\beta$ -isostrophanthidic acid. And since permanganate gave directly only  $\beta$ -isostrophanthic acid,  $\beta$ -isostrophanthidic acid must exist in solution either in the aldehydic or the oxidic form. The aldehyde and secondary  $\text{OH}^{\text{III}}$  must, therefore, be *cis* to one another, although upon adjacent rings. In  $\alpha$ -isostrophanthidic acid, the configuration is *trans*, and here an oxidic form is impossible. Although the  $\alpha$  acid was prepared in the presence of an excess of reagent, a special effort was made to oxidize it further with hypobromite to see whether an  $\alpha$ -monobasic dilactone acid could be prepared from it. All such experiments resulted in recovery of unchanged material. This shows the specific behavior of hypobromite for the oxidic form and is in agreement with the observations made in the previous communication that the preparation of isostrophanthidic acid from saponified isostrophanthidin is an oxidation of the oxidic form of the liberated second aldehyde group to the lactone group in the resulting lactone acid. Similarly, permanganate acts only upon the aldehydic form, so that with this reagent the corresponding isostrophanthic acids resulted from either  $\alpha$ - or  $\beta$ -isostrophanthidic acid.

To test further the *cis* or *trans* character of the  $\beta$ - and  $\alpha$ -isostrophanthic acids, respectively, the attempt was made to relactonize them. When  $\alpha$ -isostrophanthic acid was dissolved in concentrated hydrochloric acid, water was not removed, but a new or third isomeric isostrophanthic acid was obtained, which we have designated as  $\gamma$ -isostrophanthic acid. This behaved in all chemical properties like the  $\alpha$  form. On the other hand,  $\beta$ -isostrophanthic acid was converted into a monobasic dilactone acid which, however, was not identical with the above described  $\beta$ -isostrophanthic lactone acid but proved to be isomeric with it. Although the expected lactonization occurred because of the *cis* configuration of carboxyl<sup>I</sup> and  $\text{OH}^{\text{III}}$ , simultaneous isomerization also took place on another center of asymmetry, which is apparently the same as that involved in the above conversion of  $\alpha$ - into  $\gamma$ -isostrophanthic acid. When the new monobasic dilactone acid, or  $\delta$ -isostrophanthic lactone acid, was saponified with strong alkali, it yielded a fourth or  $\delta$ -isostrophanthic acid. The specific rotation of this acid

differed algebraically by the same amount from the  $\beta$  acid as the difference observed between the rotations of the  $\alpha$  and  $\gamma$  acids.

The exact nature of this last isomerism has not been determined, but it apparently does not involve either carboxyl<sup>I</sup> or OH<sup>III</sup>. Curiously enough, when  $\alpha$ -isostrophanthidic acid or  $\alpha$ -isostrophanthidin was similarly treated with strong hydrochloric acid, they were recovered unchanged, which was confirmed by oxidation of the resulting substance in each case to  $\alpha$ -isostrophanthic acid. The center of asymmetry affected in the above rearrangement must, therefore, be influenced by the character of the aldehydic carbon atom (C<sup>I</sup>) of the isostrophanthidin molecule.

Finally, a comparison of the oxidic ring between the CHO group and OH<sup>I</sup> as it occurs in pseudostrophanthidin and the oxidic ring between the CHO group and OH<sup>III</sup> as it occurs in dianhydrostrophanthidin and in both  $\beta$ -isostrophanthidin and  $\beta$ -isostrophanthidic acid, shows the greater stability of the former. Only the latter exists in solution both as the aldehydic and the oxidic forms.

#### EXPERIMENTAL.

*$\beta$ -Isostrophanthidic Acid.*—10.5 gm. of  $\alpha$ -isostrophanthidic acid were refluxed in 250 cc. of 2 per cent sodium hydroxide solution for 1½ hours. After cooling, the solution was acidified to Congo red with hydrochloric acid. An amorphous precipitate was soon followed by crystalline material. After standing overnight, the solid material was collected with water and sucked dry. This substance was then dissolved in a minimum volume of concentrated aqueous ammonia, and the solution was treated with several volumes of 20 per cent ammonium sulfate solution. An ammonium salt slowly crystallized on standing and rubbing. After several days, the salt was collected and washed with 20 per cent ammonium sulfate and then dissolved in 50 cc. of water. To the hot solution, 20 per cent of solid ammonium sulfate was added, causing the rapid crystallization of the salt. After cooling, this was collected. An aqueous solution of the salt was treated with hydrochloric acid, which caused the immediate separation of the free acid as arborescent aggregates of minute, short, stout crystals. After collecting with water, the yield was 1.8 gm. When recrystallized from alcohol, it slowly separated as sparingly soluble, lustrous

leaflets which softened above  $160^{\circ}$  and slowly melted together, finally frothing up between  $175^{\circ}$  and  $180^{\circ}$ . The melting point depended greatly upon the rate of heating. Aside from the more sparing solubility of its ammonium salt and the lower melting point, it resembles very closely the  $\alpha$  acid.

$$[\alpha]_D = -14^{\circ} \text{ (c = 1.067 in 95 per cent alcohol).}$$

For analysis, the substance was dried at  $100^{\circ}$  and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{23}\text{H}_{32}\text{O}_7$ .	Calculated.	C 65.68, H 7.68.
	Found.	" 65.58, " 8.00.

On acidification of the mother liquor from the above ammonium salt, amorphous material was precipitated which was followed by a copious crystallization of the unchanged  $\alpha$ -isostrophanthidic acid.

*Oxidation of  $\beta$ -Isostrophanthidic Acid with Permanganate to  $\beta$ -Isostrophanthic Acid.*—0.5 gm. of the above  $\beta$  acid was dissolved in 10 cc. of water and a few drops of ammonia. The solution was carefully treated with 3 cc. of 5 per cent potassium permanganate or in slight excess over 1 mol of O. A few cc. of saturated salt solution were added to coagulate the  $\text{MnO}_2$ . Acidification of the filtrate with acetic acid caused an immediate precipitation of delicate needles. The yield was 0.43 gm. After recrystallization from dilute alcohol, it melted with effervescence at  $280^{\circ}$  and proved identical in all its properties with  $\beta$ -isostrophanthic acid.

$[\alpha]_D^{25} = -24^{\circ}$	(c = 1.053 in methyl alcohol).
$\text{C}_{23}\text{H}_{32}\text{O}_8$ .	Calculated. C 63.26, H 7.40.
	Found. " 63.55, " 7.45.

*Oxidation of  $\beta$ -Isostrophanthidic Acid with Hypobromite to  $\beta$ -Isostrophanthic Lactone Acid.*—0.5 gm. of the  $\beta$  acid was carefully added to a solution of 0.25 gm. of bromine in 10 cc. of 2 per cent sodium hydroxide solution, and the mixture was allowed to stand for 1 hour. Acidification with hydrochloric acid caused liberation of the excess of bromine and an immediate gummy precipitate which gradually hardened as voluminous needles separated. Recrystallized from dilute alcohol, it formed minute platelets which melted at  $255\text{--}257^{\circ}$  and agreed, except in water content, in all

properties with the previously described acid  $C_{23}H_{30}O_7$ .<sup>3</sup> On one occasion, a melting point of 163–165° was observed, but this changed again to the higher figure on recrystallization. To this may be added the observation that this dilactone is not affected at ordinary temperature by chromic acid in acetic acid solution.

$$[\alpha]_D = -65^\circ \text{ (c = 1.01 in 95 per cent alcohol).}$$

*Air-Dry Substance.* Dried at 100° and 15 mm. over  $H_2SO_4$ .

$C_{23}H_{30}O_7 \cdot H_2O$ . Calculated.  $H_2O$  4.13.

Found. " 3.71.

*Anhydrous Substance.*

$C_{23}H_{30}O_7$ . Calculated. C 65.99, H 7.23.

Found. " 66.10, " 7.34.

When an attempt was made to oxidize  $\alpha$ -isostrophanthidic acid by the above procedure, it was recovered unchanged, showing that, contrary to the  $\beta$  acid, it does not exist in the oxidic form, the only form upon which hypobromite appears to react. From 1 gm. of  $\alpha$  acid, 0.55 gm. of recrystallized acid was recovered which melted at 220° with effervescence.

*Air-Dry Substance.* Dried at 100° and 15 mm. over  $H_2SO_4$ .

$C_{23}H_{32}O_7 \cdot H_2O$ . Calculated.  $H_2O$  4.27.

Found. " 4.47.

*Anhydrous Substance.*

$C_{23}H_{32}O_7$ . Calculated. C 65.68, H 7.68.

Found. " 65.95, " 7.78.

*Saponification of  $\beta$ -Isostrophanthic Lactone Acid.*—The behavior of  $\beta$ -isostrophanthic lactone acid towards 0.1 N sodium hydroxide solution has been previously described. Saponification of both lactone groups was accomplished as follows. 0.3083 gm. of the dilactone was refluxed for 3 hours in 5.5 cc. of N sodium hydroxide solution and then titrated back against phenolphthalein. Calculated for 3 equivalents, 2.21 cc. Found, 2.13 cc. The resulting solution when acidified with hydrochloric acid yielded needles which after recrystallization from dilute alcohol melted at 275–277° and proved to be identical with  $\beta$ -isostrophanthic acid.

$$[\alpha]_D = -27.5^\circ \text{ (c = 1.02 in methyl alcohol).}$$

$C_{23}H_{32}O_8$ . Calculated. C 63.26, H 7.40.

Found. " 63.23, " 7.56.

<sup>3</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lxi, 396.

*$\gamma$ -Isostrophanthic Acid.*—A solution of  $\alpha$ -isostrophanthic acid in 10 parts of hydrochloric acid (1.19) was left for 1 hour at 20°. On dilution, an immediate copious crystallization of six-sided platelets occurred. The collected acid was recrystallized from dilute alcohol, forming bundles of needles and spears. The melting point varied greatly with different preparations or when the same preparation was recrystallized. Most frequently, the substance was found to melt with effervescence at 231–232°, although 240–242° and 248° were also observed. No attempt was made to determine the cause of this variation, which has been noted so frequently with this class of substances. In this case it is possibly referable to varying amounts of solvent, since one preparation containing 2 mols of water effervesced at 227–230° and one containing 1 mol at 248°.

The substance was also obtained by heating at 100° for 2 hours the  $\alpha$  acid dissolved in 50 parts of 10 per cent hydrochloric acid and 20 parts of alcohol. It is easily soluble in alcohol, acetone, and acetic acid. It may be recrystallized from hot water, forming characteristic hexagonal platelets. It gives gradually a permanent purple-red color in  $\text{H}_2\text{SO}_4$  like the  $\alpha$  and  $\beta$  acids.

$[\alpha]_D = +90^\circ$  ( $c = 1.000$  in 95 per cent alcohol) for the air-dry substance.

0.0840 gm. of anhydrous substance on direct titration against phenolphthalein required 3.75 cc. of 0.1 N NaOH. Calculated for 2 equivalents, 3.85 cc.

*Air-Dry Substance.* Dried at 100° and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{23}\text{H}_{32}\text{O}_8 \cdot \text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  3.97.

Found. " 4.12.

*Anhydrous Substance.*

$\text{C}_{23}\text{H}_{32}\text{O}_8$ . Calculated. C 63.26, H 7.40.

Found. " 63.52, " 7.31.

*$\gamma$ -Isostrophanthic Dimethyl Ester.*—The acid was treated in acetone solution with diazomethane. It formed needles from methyl alcohol which melted at 227°.

$[\alpha]_D = +120^\circ$  ( $c = 1.04$  in pyridine).

$\text{C}_{25}\text{H}_{34}\text{O}_8$ . Calculated. C 64.66, H 7.82.

Found. " 64.59, " 8.14.

*γ-Isostrophanthonic Dimethyl Ester.*—This was prepared in practically quantitative yield by oxidation of the above ester exactly as described for the  $\alpha$  isomer in the preceding communication. It formed narrow platelets and needles from methyl alcohol which melted slowly at  $235^{\circ}$ .

$$[\alpha]_D = +106^{\circ} \text{ (c = 1.05 in pyridine).}$$

$C_{28}H_{34}O_8$ .	Calculated.	C 64.90, H 7.41.
	Found.	" 64.96, " 7.26.

*γ-Anhydroisostrophanthonic Dimethyl Ester.*—Dehydration of the above keto ester occurred readily as previously given for the  $\alpha$  isomer. Recrystallized from methyl alcohol, it formed small, stout prisms and rhombs which melted, not sharply, from  $168$ – $171^{\circ}$ .

$$[\alpha]_D = +160^{\circ} \text{ (c = 1.100 in pyridine).}$$

$C_{26}H_{32}O_7$ .	Calculated.	C 67.53, H 7.26.
	Found.	" 67.25, " 7.11.

*δ-Isostrophanthic Lactone Acid.*—3 gm. of  $\beta$ -isostrophanthic acid were shaken in 30 cc. of hydrochloric acid (1.19) at room temperature ( $25^{\circ}$ ) for  $1\frac{1}{2}$  hours, during which solution gradually occurred. When diluted with water, an amorphous precipitate fell which mostly crystallized on longer standing for several days. The collected acid was suspended in a few cc. of water and carefully treated with a slight excess of ammonia. The salt crystallized during the process. The volume was increased by the addition of 20 per cent ammonium sulfate solution, and after standing the salt was collected. Acidification of a warm solution of the salt gave an emulsion which slowly crystallized when seeded. The collected acid was recrystallized by careful dilution of the concentrated methyl alcoholic solution. A crust of long prisms and tables slowly deposited which melted at  $230$ – $231^{\circ}$  with effervescence. Like  $\beta$ -isostrophanthic lactone acid, it dissolves in sulfuric acid with a very slowly developing straw color which deepens only on long standing, finally becoming a brown-olive.

$$[\alpha]_D^{27} = +29^{\circ} \text{ (c = 0.994 in pyridine).}$$

$C_{29}H_{36}O_7$ .	Calculated.	C 65.99, H 7.23.
	Found.	" 66.01, " 7.34.

*δ-Isostrophanthic Acid*.—The previous dilactone was refluxed for 3 hours in 4 per cent sodium hydroxide solution. On acidification to Congo red, a resin slowly deposited which gradually crystallized on standing. Recrystallized from hot water in which it is appreciably soluble, it separated as slender, flat needles which melted at 209–210° with effervescence. It is more soluble than the *β* acid. In sulfuric acid, it gave the same gradually developing purple-red color shown by the isomeric acids.

$[\alpha]_D^{27} = +72^\circ$  ( $c = 1.05$  in methyl alcohol).

$C_{22}H_{22}O_8$ . Calculated. C 63.26, H 7.46.

Found. " 62.75, " 7.57.





**A MICRO METHOD FOR THE QUANTITATIVE DETERMINATION OF CARBON DIOXIDE IN BLOOD AND OTHER SOLUTIONS, AND SOME OBSERVATIONS ON THE EFFICIENCY OF PARAFFIN OIL AS A MEANS OF KEEPING CARBON DIOXIDE IN SOLUTION.**

By DANIEL RAFFEL.

(*From the Physiological Institute, University of Copenhagen, Copenhagen, Denmark.*)

(Received for publication, April 28, 1927.)

The principle of this method is the same as that of Krogh and Rehberg<sup>1</sup> for the determination of CO<sub>2</sub> in air; *viz.*, the absorption of the gas in a known quantity of Ba(OH)<sub>2</sub> which is then titrated. The CO<sub>2</sub> in the solution is liberated both by the addition of acid and heating to 100°C. while a constant stream of CO<sub>2</sub>-free air is passed through the mixture.

The method has been tried and found satisfactory for the determination of CO<sub>2</sub> in solutions of inorganic carbonates, blood serum, and whole blood. The quantity of the solution necessary for an analysis is determined by the quantity of CO<sub>2</sub> in the solution. Since the method is accurate for between 0.1 and 0.5 cc. of CO<sub>2</sub>, the quantity of solutions which is certain to contain an amount of CO<sub>2</sub> between these limits should be chosen.

*Apparatus.*

The apparatus (Fig. 1) used for this method is constructed as follows: The tube in which the CO<sub>2</sub> is liberated (A) is made of an ordinary test-tube to which a piece of narrow glass tubing 0.3 cm. in diameter with a bulb of about 7 cc. capacity is blown. The tube in which the CO<sub>2</sub> is absorbed (B) is made as shown in Fig. 1. It is a U-shaped glass tube 0.8 cm. in diameter. In the upper

<sup>1</sup> Krogh, A., and Rehberg, P. B., *Skand. Arch. Physiol.*, 1926, liv, 167.

surface of the bottom of the U-tube a stop-cock is provided which is used for emptying this part of the apparatus. Near the top of one arm of the tube a short piece of tubing of the same bore is attached. To the end of the other arm is blown a bulb about 2.5 cm. in diameter, blown on the end of an 8 cm. piece of tubing 1.5

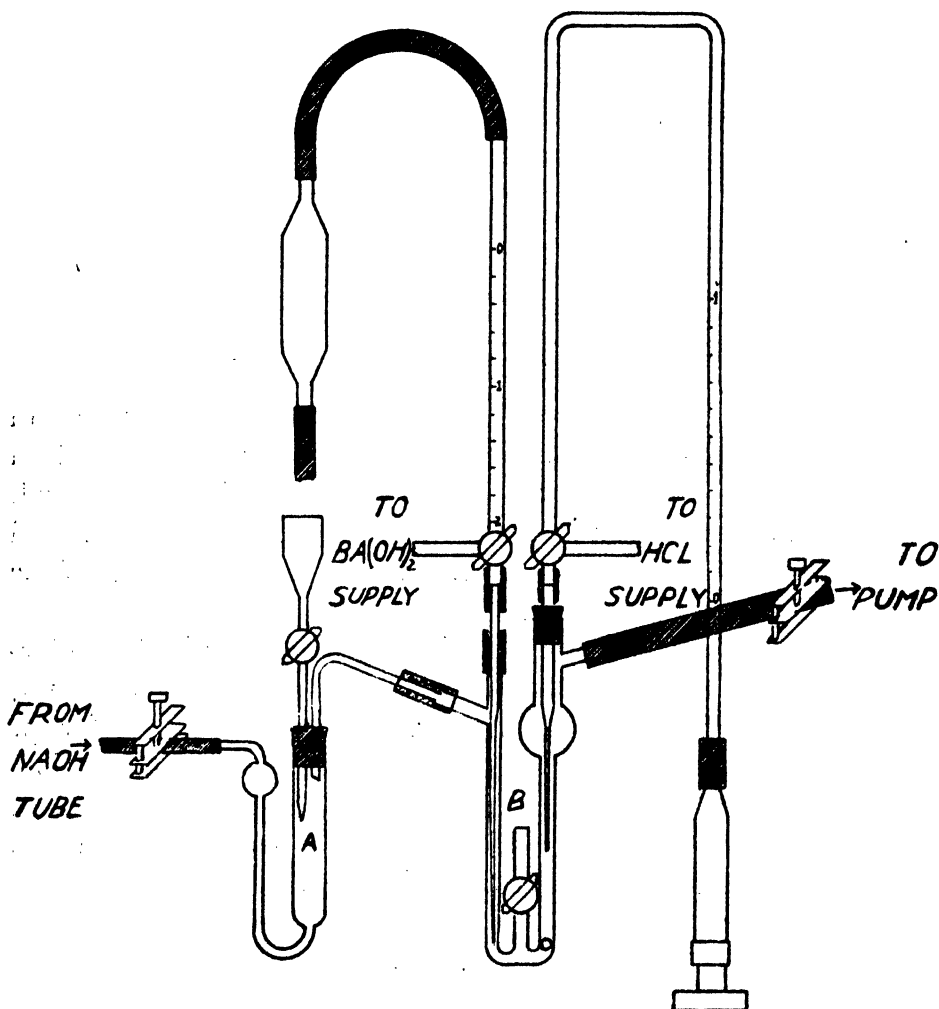


FIG. 1.

cm. in diameter with an arm of glass tubing 0.8 cm. in diameter attached 4 cm. from the top. A glass bead, just large enough to remain near the bottom of the tube, must be inserted in the tube in order to break the air into small bubbles. These should not be larger than 0.2 cm. in diameter.

The acid used for the titration is measured in a graduated 1 cc. pipette which is attached by means of very heavy vacuum tubing to a modified micro titration syringe. The other end is attached to a piece of capillary glass tubing which ends at a capillary three-way stop-cock. From here the acid is conducted into the solution to be titrated through a capillary glass tube which is drawn out until it is very fine. The third opening from the stop-cock is connected by capillary tubing to the Wolff bottle in which the acid is kept.

The  $\text{Ba}(\text{OH})_2$  is measured in a graduated 2 cc. pipette which is attached at one end to a three-way stop-cock, and at the other to a piece of rubber tubing through which the pipette is filled by suction. A tube containing soda-lime is inserted in the rubber tubing to protect the pipette and its content from the  $\text{CO}_2$  in the

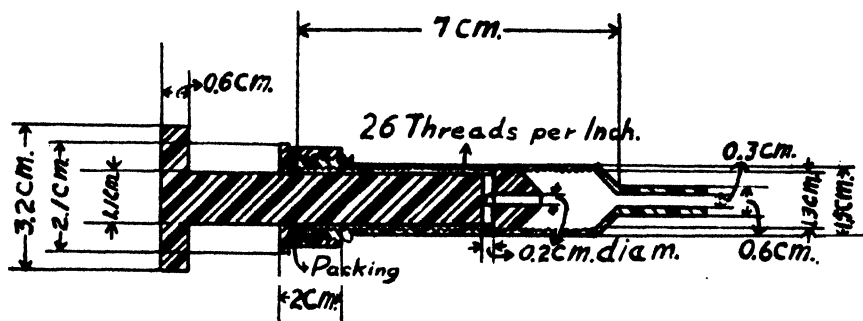


FIG. 2. Micro titration syringe.

atmosphere. The openings to the stop-cock are connected respectively with capillary tubing leading to the Wolff bottle in which the  $\text{Ba}(\text{OH})_2$  is kept, and to a piece of glass tubing 0.3 cm. in diameter which is drawn out to 0.1 cm. in diameter and reaches to the bottom of the tube in which absorption takes place. This tube is drawn out to a fine point at its lower end.

The micro titration syringe must be air-tight so that the negative pressure in the apparatus does not cause air to be drawn in through the syringe. It is constructed of steel as shown in Fig. 2. The cylinder is a steel tube 1.5 cm. in diameter and 0.2 cm. thick. At the front end it tapers to the same size as the pipette which is to be attached to it. The last 1.5 cm. at the other end is made 1.7 cm. in diameter. On the inner surface and on the outer surface of the thicker end threads are cut. The piston of

the syringe has threads which fit in the threads on the inner surface of the cylinder. From the end of the piston a hole is cut which reaches to the shaft of the piston where it connects with two other channels cut at right angles through the shaft. This allows the mercury with which the syringe is to be filled to occupy continuously all the space within the cylinder. At the other end of the shaft a disk 3.2 cm. in diameter is attached, which is the part turned in order to fill or empty the burette. A collar containing packing fits over the shaft of the piston and screws on to the end of the cylinder. The syringe is assembled and filled under mercury. Then some of the mercury is forced into a piece of glass tubing which is attached to the syringe. This is then attached to an aspirator pump and shaken violently to expel all the air from the syringe. When, upon disconnecting the aspirator, no change is observed in the height of the mercury column, all the air has been removed and the syringe is ready for use.

The parts of the apparatus described above are all suspended from a single clamp stand making the whole very compact and requiring very little space. The apparatus is connected as is shown in Fig. 1. Tubes A and B are connected by a piece of narrow glass tubing which enters Tube A through a two-hole rubber stopper. In the other hole is placed a funnel with a stop-cock. Tube A is connected to an absorption tube containing a 50 per cent solution of  $\text{NaOH}$ . A form found very effective is a series of ten bulbs through which the air is slowly bubbled. Tube B is connected to an aspirator by means of which the stream of air is drawn through the apparatus.

### *Solutions.*

The  $\text{HCl}$  solution used for titrating is made 0.0894 N because at this concentration a volume of solution is chemically equivalent to the same volume of  $\text{CO}_2$  gas at normal temperature and pressure, and therefore the volume of  $\text{CO}_2$  is obtained directly.

The  $\text{Ba}(\text{OH})_2$  solution is made by diluting saturated  $\text{Ba}(\text{OH})_2$  to 10 times its volume. The exact normality of this solution is of no importance, but this concentration was chosen as 2 cc. require approximately 0.7 cc. of acid to neutralize this amount.

The indicator used is thymolphthalein because it gives a very

sharp end-point at a pH at which there can be no liberation of  $\text{CO}_2$ . The solution used is 0.1 gm. dissolved in 50 cc. of alcohol and 50 cc. of water.

The indicator is added to the  $\text{Ba}(\text{OH})_2$  when the latter is placed in the Wolff bottle. 25 cc. of indicator are added to each 400 cc. of  $\text{Ba}(\text{OH})_2$  solution.

The acid used to liberate the  $\text{CO}_2$  is 0.1 N HCl.

### *Technique.*

The burette to contain the  $\text{Ba}(\text{OH})_2$  is connected as shown in Fig. 1. It is filled by sucking through the rubber tubing attached to the pipette until the pipette is full. Then by turning the stop-cock the  $\text{Ba}(\text{OH})_2$  is allowed to run out of the pipette into the narrow glass tubing. The acid burette is filled by forcing the mercury as far as the stop-cock. Then the stop-cock is turned to connect the supply of acid to the narrow tube. By means of a piece of rubber tubing placed over the drawn out end of the tube acid is sucked into the latter. The stop-cock is then turned to connect the supply of acid to the pipette. By means of the micro titration syringe the burette is filled. The air is forced out of the burette which is then refilled and is ready for use.

The apparatus is then assembled as is shown in Fig. 1 and is tested to see that it is air-tight. This is done by closing the entrance to the absorption apparatus containing NaOH and drawing the air stream through the apparatus rapidly. If after a time no bubbles pass through the liquid in Tube B, the apparatus is tight. If however, the stream of bubbles does not cease to pass through the liquid, there is a leak which must be located. As soon as the apparatus is air-tight, some  $\text{Ba}(\text{OH})_2$  is let down into Tube B and air is drawn through the apparatus for 10 minutes to expel any  $\text{CO}_2$  that may be present. The fluid in the tube must come up to the end of the tube through which the acid enters Tube B. The  $\text{Ba}(\text{OH})_2$  solution is then brought to zero on the pipette. Then acid is added slowly until the last trace of color has disappeared. During the addition of the acid the rubber tube leading to the aspirator is frequently squeezed in order to force the fluid back into the other arm of Tube B. In this way all of the wet parts of the tube are neutralized.

A very small amount of  $\text{Ba}(\text{OH})_2$  (0.005 cc.) is allowed to enter the tube and if the color had just left the fluid, it should again be perceptible from the addition of this small amount of  $\text{Ba}(\text{OH})_2$ . If the color is not restored, more  $\text{Ba}(\text{OH})_2$  must be let into the tube and the operation repeated. When the addition of 0.005 cc. of  $\text{Ba}(\text{OH})_2$  restores the color to the fluid, the remainder of the 2 cc. is allowed to flow into the tube. Air is drawn through the apparatus during the whole of the process. The acid pipette is then filled to the zero mark by means of the micro titration syringe. Acid is then slowly added to the solution until it is just colorless. The mixing of the solution is facilitated by squeezing the tubing to the aspirator and thus forcing the fluid up and down in the tube and once more neutralizing all wet parts of the tube. The two screw clamps are then tightened and most of the fluid is drawn out of the apparatus. The amount of acid required for this titration is determined several times. A maximum variation of 0.5 per cent is usual for such a series of titrations. After the titration has once been done this way for a flask of solution, only a single titration need be made on days when the apparatus is to be used in order so see that the solutions remain constant. This titration is the control for the determinations to be made.

When the apparatus has not been used for a long period, it is advisable to make a control determination; *i.e.*, after the 2 cc. of  $\text{Ba}(\text{OH})_2$  have been let into the tube, air is drawn through the apparatus for 30 minutes before the titration is completed. This result should check with the previous ones. If it does not it indicates either that there is a leak in the apparatus or that the  $\text{NaOH}$  is no longer sufficiently concentrated.

1 cc. of octyl alcohol is placed in the funnel and as soon as the 2 cc. of  $\text{Ba}(\text{OH})_2$  are introduced into Tube B, the alcohol is allowed to run into Tube A until the last of it is just about to enter the stop-cock. About 3 cc. of paraffin oil are then put into the funnel. The solution to be tested is introduced into the funnel, by a pipette, under the paraffin oil. The solution is allowed to drop slowly into Tube A until a small quantity of the paraffin oil above it has gone through the stop-cock. Then 2 cc. of about 0.1 N  $\text{HCl}$  are added under the paraffin oil and allowed to drop until the last of the oil is about to pass into the stop-cock.

The water bath (heated previously) is then placed under Tube

A and the apparatus is allowed to run for 25 minutes. The air should always be drawn through at a rate of 7 to 9 cc. per minute. At the end of 25 minutes the mixture is titrated as in the controls.

The reading obtained from the acid pipette is subtracted from the control titration already obtained. The result is the number of

TABLE I.

$N/30 \text{ Na}_2\text{CO}_3$ .	$\text{CO}_2$ present.	$\text{CO}_2$ obtained.	Error.
cc.	cc.	cc.	per cent
0.25	0.093	0.093 0.095 0.095 0.094	
Average.....		0.094	1.1
0.50	0.187	0.190 0.186 0.189 0.190	
Average.....		0.189	1.1
1.00	0.373	0.371 0.374 0.375 0.376 0.374	
Average.....		0.374	0.3
1.34	0.500	0.502 0.498 0.493 0.492	
Average.....		0.496	0.8

cc. of  $\text{CO}_2$  at normal temperature and pressure contained in the sample examined.

After the control titration has been made analyses can be made every 45 minutes. It is quite a simple matter to have two or three sets of apparatus using the same solutions, so that one can make simultaneous determinations.



*Accuracy of the Method.*

This method has been found to be accurate for samples containing from 0.100 to 0.500 cc. of CO<sub>2</sub>. In all tests the maximum deviation was found to be equal to or less than 2 per cent and

TABLE II.  
*Analyses of Rabbit Sera.*

A. 1 cc. serum.			B. 0.5 cc. serum.		
Control.	Acid in burette.	CO <sub>2</sub>	Control.	Acid in burette.	CO <sub>2</sub>
cc.	cc.	cc.	cc.	cc.	cc.
0.719	0.209	0.510	0.697	0.486	0.211
0.719	0.209	0.510	0.697	0.488	0.209
0.719	0.207	0.512	0.697	0.490	0.207
0.719	0.206	0.513	0.697	0.489	0.208
0.719	0.206	0.513	0.697	0.487	0.210
0.719	0.205	0.514	0.697	0.486	0.211
0.719	0.206	0.513			
0.719	0.200	0.519			
0.719	0.207	0.512			
Average.....		0.513			0.209

TABLE III.  
*Analyses of Rabbit Blood (0.5 Cc.).*

Control.	Acid in burette.	CO <sub>2</sub>	Control.	Acid in burette.	CO <sub>2</sub>
cc.	cc.	cc.	cc.	cc.	cc.
0.698	0.425	0.273	0.698	0.473	0.225
0.698	0.424	0.274	0.698	0.477	0.221
0.698	0.422	0.276	0.698	0.476	0.222
0.698	0.426	0.272			
Average.....		0.274			0.223

when many determinations were made the average deviation was found to be 0.4 per cent.

The method was tried first on a known solution of N/30 Na<sub>2</sub>CO<sub>3</sub>. The results obtained are given in Table I.

The method was then tried on rabbit sera, 0.5 and 1 cc. being used. Typical results are shown in Table II.

Whole blood of rabbits was then analyzed by this method and yielded similar results to those obtained from serum and inorganic solutions. Table III gives typical results obtained from 0.5 cc. of rabbit blood.

A comparison was then made of the results obtained by this method and those obtained by pumping the dissolved gases out of serum and then analyzing the gas mixture. The result of this experiment is as follows:

<i>Analyses of 0.5 Cc. of Serum.</i>		
Control.	Acid in burette.	CO <sub>2</sub> in serum.
cc.	cc.	cc.
0.661	0.278	0.383
0.661	0.278	0.383
0.661	0.273	0.388
Average.....		0.385 = 77.0 vols. per cent.

The analyses of the gases pumped out of 20.5 cc. of serum gave 16.59 cc. of CO<sub>2</sub> at 17.7°C. and 764.90 mm. When this is corrected to 0°C. and 760 mm. it is 15.61 cc. of CO<sub>2</sub> in 20.5 cc. of serum or 76.1 volumes per cent. The difference in results obtained is 0.9 volumes per cent, or a difference of 1.2 per cent of the total.

#### *Observations Made on Sera Left under Paraffin Oil.*

Some observations were made on sera which was left for some time under paraffin oil, in order to ascertain whether or not any measurable change in the CO<sub>2</sub> content takes place. It was found that the results depend on (a) the quantity of serum used and (b) on the CO<sub>2</sub> content of the serum. In the case of serum with normal CO<sub>2</sub> content (50 to 60 volumes per cent) the change was found to be very small, amounting to 2 per cent or less in a day. However when there is a large excess of CO<sub>2</sub> in the serum, *e.g.* 80 volumes per cent, the loss in 1 day may amount to 10 per cent.

Results of some experiments are given below.

*Experiment 1.*—About 40 cc. of serum were taken and placed under paraffin oil. Analyses were made on the 1st, 2nd, 4th, and 6th days. The values given are the means of four determinations except that of the 6th day when there was only enough serum for one analysis.

Day.	CO <sub>2</sub> in 1 cc. serum. cc.
1	0.542
2	0.531
4	0.523
6	0.503

*Experiment 2.*—This experiment was the same as the previous one but analyses were made only on the 1st, 2nd, and 6th days.

Day.	CO <sub>2</sub> in 1 cc. serum. cc.
1	0.561
2	0.561
6	0.533

*Experiment 3.*—This experiment was made in the same way but extended only over 2 days.

Day.	CO <sub>2</sub> in 1 cc. serum. cc.
1	0.513
2	0.508

*Experiment 4.*—In this experiment about 60 cc. of serum were obtained. They were then placed under paraffin oil and 5, 10, and 15 cc. samples were taken and placed under 10 cc. of paraffin oil in test-tubes and left until the next day. The remainder was analyzed and found to contain 0.386 cc. of CO<sub>2</sub> per cc. of serum. The analyses of the other samples after 24 hours yielded the following.

Sample. cc.	CO <sub>2</sub> cc.	CO <sub>2</sub> lost. cc.
5	0.378	0.003
10	0.382	0.004
15	0.386	0.000

These experiments show that in the case of normal or nearly normal serum, the loss of CO<sub>2</sub> is very small when the sera are kept under paraffin oil. It never exceeds 2 per cent per day.

In some experiments connected with another investigation, some sera containing extraordinary quantities of CO<sub>2</sub> (80 to 150 volumes per cent) were found to lose as much as 10 per cent of it in the course of 1 day.

#### SUMMARY.

A method is described for the determination of small amounts of CO<sub>2</sub> in blood and other solutions. It is accurate for amounts

between 0.1 and 0.5 cc. of  $\text{CO}_2$ , with an error not exceeding 1.1 per cent.

The method is based on the absorption of the  $\text{CO}_2$  in  $\text{Ba}(\text{OH})_2$  which is then titrated.

By the aid of this method it was found that normal serum kept under paraffin oil loses only 2 per cent or less of its  $\text{CO}_2$  in the course of 24 hours. However, when large quantities of  $\text{CO}_2$  are present the loss may amount to 10 per cent of the  $\text{CO}_2$  contained in the serum.

I wish to acknowledge the invaluable assistance that I have received from Professor V. Henriques in this work.



# STUDIES ON URINARY ACIDITY, AND A METHOD FOR ELECTROMETRIC TITRATION OF URINE.

BY SERGIUS MORGULIS AND W. R. HAMSA.

*(From the Department of Biochemistry, University of Nebraska, College of Medicine, Omaha.)*

(Received for publication, June 13, 1927.)

The titratable acidity of urine represents the quantity of acid eliminated through the kidneys uncombined with base. This quantity in conjunction with the acid neutralized by ammonia should give a measure of the total base economy in metabolism. Since the appearance of Folin's classical studies on urine acidity (1) the determination is very generally carried out by a procedure devised by him and recommended in various laboratory manuals. The principal feature in this determination is the titration of the urine with 0.1 N NaOH to the first definite change of color of phenolphthalein in the presence of an excess of neutral potassium oxalate. The titration aims to determine the total amount of acid phosphate. Henderson (2), however, has pointed out that the work of the kidney in maintaining the physiological reaction of the blood is indicated more exactly by the alkali required to titrate the urine to that reaction (pH 7.4). Henderson and Adler (2) performed this titration with neutral red to indicate the end-point. In the work here reported the principle of titration to pH 7.4 has been employed, but the end-point has been determined by the quinhydrone electrode, employed as described by Meeker and Oser (3), according to the method of Klopsteg (4): one electrode is immersed in a solution of known pH, and the other is immersed in the unknown solution, which is titrated until the potential difference, indicated by a galvanometer, disappears. The essential difference between our procedure and that of Meeker and Oser, was that, since they wished to determine the pH of the urine, they titrated the buffer solution till its pH equalled that of the urine. We have reversed the procedure, and have titrated the urine until the pH equalled that of the known buffer solution (7.4).

The titration is simple and reliable, demanding only a few minutes. The titration proceeds rapidly and is extremely sensitive, even a fraction of a drop being definitely registered by an oscillation of the galvanometer.

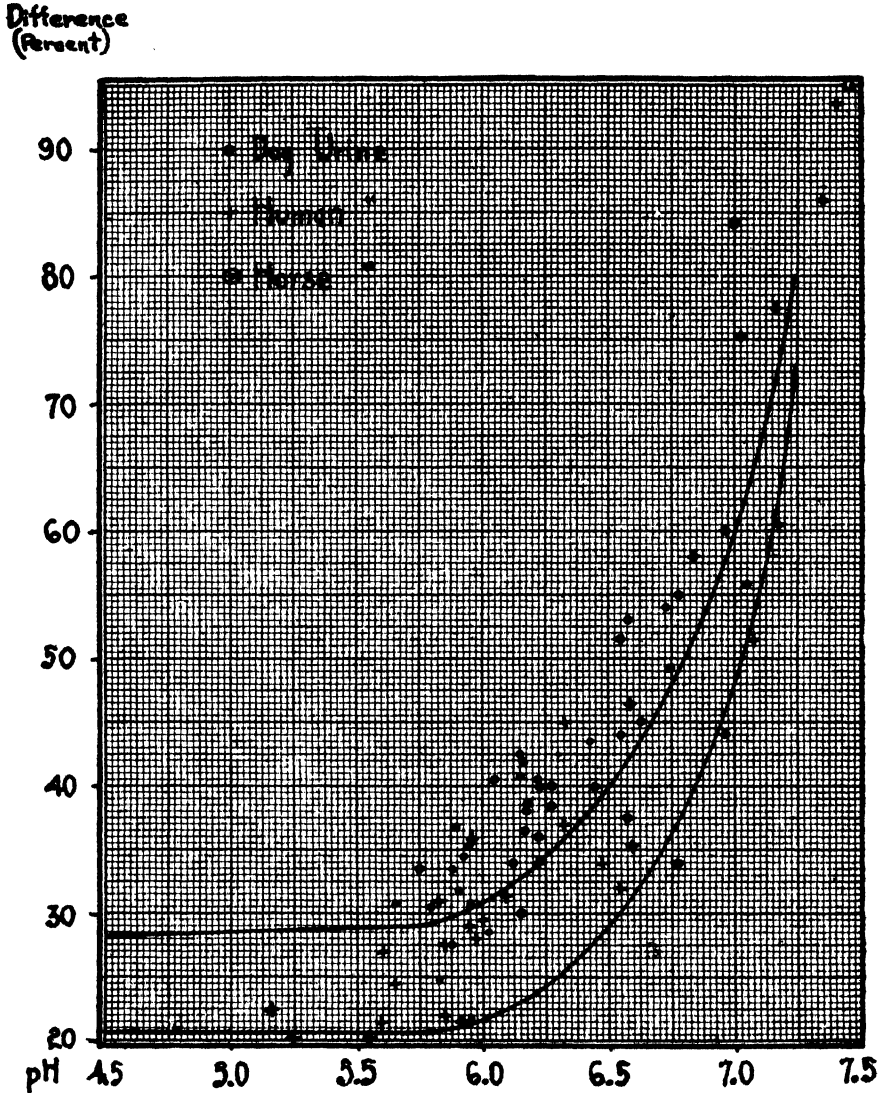


FIG. 1.

If the urinary acidity was studied by the method just described and by Folin's method it soon became apparent that the results obtained by our procedure are not only almost always lower (only

once in a very large number of determinations were the titration results by our procedure higher) as might have been anticipated, but it was found also that the magnitude of the variation between the two types of titration was a function of the pH of the urine. If the difference between the two determinations (expressed in per cents) is indicated on ordinates, the abscissæ representing the pH of the corresponding urine sample, it is noted that the points arrange themselves in such a manner that a definite curve can be traced through them, rising slowly between pH 5 and 6, then much more rapidly between pH 6 and 7, and becoming almost perpendicular at pH above 7.0. In other words, the degree of discrepancy

TABLE I.

pH of solution.	Cc. of 0.1 N NaOH used per 100 cc.					Cc. 0.1 N free organic acid at pH 7.4. (e)	Per cent difference between (d) and:	
	Phenolphthalein titration to:		Electrometric titration to pH 7.4.				(a)	(b)
	pH 7.9 (a)	pH 8.4 (b)	Theoretical. (c)	Found. (d)	Difference. (d - c)			
4.66	53.9	60.0	39.6	43.0	+3.4	4.0	20	28
4.88	53.0	58.0	39.3	41.7	+2.4	2.5	21	28
5.72	44.5	50.0	36.2	36.0	-0.2	0.4	19	29
5.96	41.6	47.4	33.7	32.5	-1.2	0.2	22	32
6.46	32.0	37.0	24.3	23.3	-1.0		27	37
6.96	19.2	24.8	11.0	10.4	-0.6		46	58
7.22	12.0	17.5	3.8	3.7	+0.1		69	79
7.50	6.2	11.7						

between the two determinations increases with the falling H ion concentration of the urine.

To gain some understanding of the cause of this difference we prepared mixtures containing  $\text{KH}_2\text{PO}_4$ ,  $\text{NH}_4\text{Cl}$ , and lactic acid each 0.05 M, approximating very closely the concentration of phosphate, ammonia, and organic acid in the diluted dog urine samples with which we have been working. The mixtures were adjusted to different pH by adding varying amounts of N NaOH. Aliquot portions from each were then titrated for free acidity by our method and that of Folin. The titration with phenolphthalein was carried out in two stages: to a first recognizable pink at pH 7.9 and to a definite red at pH 8.4. The electrometric titration



was to the pH 7.4, as in the case of the urines. Table I contains the results of these titrations, calculated on the basis of 100 cc., from which it is obvious, first, that the electrometric titration gives practically the theoretical amounts of base necessary to effect the change in proportion of monobasic and dibasic phosphates from the pH of the mixture to the pH of the reference electrode (pH 7.4); secondly, that the organic acid, except at pH below 5.0, plays a relatively insignificant part in the titration of the free acidity; and, finally, that the difference between the titration values of mixtures of various pH by the electrometric and by the indicator method is essentially like that observed in urines. In Fig. 1 the curves corresponding to the results obtained from the titration of these known mixtures are drawn in (the upper curve represents the difference when the titration was to pH 8.4 and the lower curve to pH 7.9), and it is clear that the results from the study of various urines (human, dog, horse) range themselves so closely along these curves as to suggest that the same factors may be responsible in both instances. (It should be noted that similar curves were obtained with pure phosphate solutions of different pH.)

In his paper on the acidity of the urine Folin says:

"It has been very generally assumed that the acidity of urine is almost wholly due to the presence of monobasic phosphate, and that the organic acids of normal urine play but a very subordinate rôle. The extent to which the actual acid H ions of urine are derived from phosphoric acid depends on the amounts of the organic acid present, since these are in general much the weaker acids and therefore combine to a correspondingly smaller extent with the bases present."

The results of the experiments here reported would seem to indicate that probably the exact reverse holds true. The nature of the various organic acids of urine is but imperfectly known, but the few acids definitely known are more strongly acid than  $\text{BH}_2\text{PO}_4$ , and at the usual pH of normal urine should be present largely in the form of their salts. In Fig. 2 the curves are plotted for five representative organic acids, showing the relative proportions of free acid at various pH. At the average pH of 6.1 of human urine only about 5 per cent of  $\beta$ -hydroxybutyric acid is in the free state. Our measurements of the pH of dog urine give on the

average a higher pH of 6.4, and we have never encountered a normal dog urine more acid than pH 5.50. Schaefer and Schmidt (5) determined the pH of 100 human urines using the quinhydrone electrode, and the average from their determinations is pH 6.33. Considering the very small amount of uric acid in dog urines, at the pH of 6.4 there should be practically no free organic acid present.



FIG. 2.

To gain some insight into the nature of the organic acids, we resorted to the following procedure in the case of the dog urines studied. We determined the organic acids quantitatively by the Van Slyke-Palmer method (6). To a second similar sample of the urine filtrate used for the organic acid analysis we added only half the amount of 0.2 N HCl required for the complete titration, making up the volume with water, and determined the pH of the mixture. Since at that point half of the organic acid should be

free and half in the form of its salts, the pH will practically correspond to the pK value and, therefore, to the dissociation constant of the acids. We say advisedly "acids" since we are unquestionably dealing with a number of acids in such a mixture and the pK thus determined represents merely a mean value. To test the soundness of this procedure, determinations were carried out with solutions of lactic acid and the results thus obtained were entirely satisfactory. In normal dog urine on a mixed diet we found values of pK of 4.1 to 4.4; in the course of fasting there has been a tendency for this value to increase up to 4.7, but in advanced stages of fasting the pK value sometimes tends in the opposite direction, and has been seen to drop even as low as 3.1. If the pK values of the organic acids are taken into consideration, it does not seem probable that these acids can play a significant rôle in the measurement of the total acidity of urine. Realizing, however, the danger of drawing conclusions from a study of relatively simple mixtures, such as were used in the previous experiments, which would apply with equal force to the behavior of an intricately complex system like urine, we repeated the above experiments with certain modifications. For this purpose we started with three dog urines of the following characteristics:

	Urine 1.	Urine 2.	Urine 3.
pH.....	5.74	6.22	6.38
Phosphate.....	0.0713 M	0.0652 M	0.0635 M
Ammonia.....	0.134 "	0.034 "	0.106 "
Organic acid.....	0.0714 "	0.0375 "	0.058 "
pK of organic acids.....	4.1	4.4	4.2

Three mixtures having the same phosphate, ammonia, and organic acid concentration were then prepared from  $\text{KH}_2\text{PO}_4$ ,  $\text{NH}_4\text{Cl}$ , and lactic acid, and the pH of each was adjusted as nearly as possible to that of the above urine samples. (Unfortunately, in order to maintain the necessary volume relationship a strong NaOH solution was required for the adjustment, which explains why we failed to reproduce the urinary pH with absolute precision.) These artificial urines as well as the dog urines were now titrated according to the Folin method and by our electrometric method. The artificial urines were titrated in two phases, to pH 7.9 and to pH 8.4, the higher pH corresponding more or less closely to the pH

to which urine is titrated with phenolphthalein at the first color change.

In Table II the results of this experiment are given, calculated for 100 cc. In the artificial urine mixtures the amount of 0.1 N NaOH required to titrate electrometrically to a pH 7.4 agrees very well with the calculated amount, as was also found in the previous series. This, however, does not hold for the real urines, in spite of the fact that the concentration of phosphate, ammonia, and organic acid was the same in both (the difference in pH of the real and artificial urines is not sufficiently large to account for their different behavior). Thus, the dog urines required 8.5 to

TABLE II.  
*Cc. 0.1 N NaOH per 100 Cc.*

pH of mixture.	Calculated to pH 7.4. (a)	Electro- metric titration to pH 7.4. (b)	Differ- ence. (b - a)	Phenolph- thalein titration to pH 8.4. (c)	Differ- ence. (c - a)	Per cent difference: $100 \times (c - b)$ (c)
Dog urine.						
5.74	52.4	63.2	+11.8	95.0	+43.6	-33.5
6.22	38.0	46.5	+8.5	70.8	+32.8	-34.3
6.38	32.3	43.0	+10.7	68.6	+36.3	-37.3
Artificial urine.						
5.85	50.6	49.5	-1.1	73.0	+22.4	-32.2
6.34	34.5	33.0	-1.5	49.6	+15.1	-33.5
6.50	28.4	28.6	-0.2	47.7	+19.3	-40.0

11.8 cc. of 0.1 N NaOH more per 100 cc. to attain the pH of 7.4 (electrometric) and 32.8 to 43.6 cc. of 0.1 N NaOH to titrate to a pH of 8.4 (phenolphthalein) than the calculated amount. This represents an average increase of 27.3 cc. and is much greater than the corresponding increase for the artificial urine mixtures, where the average difference is only 18.9 cc. of 0.1 N NaOH. This increase of nearly 50 per cent in the amount of alkali needed to change the reaction from pH 7.4 to pH 8.4 is much larger than the difference of 0.1 pH between the dog urines and the artificial urines would occasion. The greater difficulty in observing the color change in a urine titration may account only partly for the recorded discrepancy.

These results suggested that urine must possess considerable buffer action which is independent of its phosphate, ammonia, or organic acid content. We studied, therefore, the buffer value of urines according to the procedure outlined by Van Slyke (7), determining the amount of alkali necessary to effect a definite change in the pH of the urine. The determinations were carried out as follows: The pH of the urine was measured by means of quinhydrone according to Meeker and Oser (3), buffer mixtures of different pH value being then substituted and the urine titrated electrometrically to this pH with 0.1 N NaOH. The buffer value was calculated from Van Slyke's formula  $\frac{\Delta B}{\Delta \text{pH}} = \beta$  for the mean pH of two determinations. The observations were made on urines from two dogs which were kept in metabolism cages and fed the same diet. The buffer values found for the urines of these two dogs are, nevertheless, very different. Generally, no less than four consecutive titrations were made on each urine sample, from the original pH to a pH 6.4, then to a pH 6.9, 7.4, and finally to a pH 8.0. It is needless to give the data *in extenso*, as this would take up too much space, but a few examples, presented in Table III, will suffice to show the trend of variation in buffer value in the two urines.

The buffer value of normal urine almost without exception increases as the titration is continued to pH 8.0, and this is demonstrated even more strikingly by the absolute amounts of alkali used up in successive stages of the titration. Thus, in the case of seven urines with an initial pH range of 5.62 to 6.09 the minimum and maximum amounts of 0.1 N NaOH used to titrate to pH 6.40 were 4.8 to 9.0 cc. per 100 cc. urine; from pH 6.40 to pH 6.90, 4.9 to 9.0 cc. were used; from pH 6.90 to pH 7.4, 4.0 to 10.0 cc. were used; and from pH 7.4 to pH 8.0 the titration required 13.0 to 15.8 cc. of NaOH. This increasing buffer value of the urine cannot, however, be ascribed to the action of phosphates since their maximum buffer value is attained at pH 6.90. To verify this point further the inorganic phosphate was determined in two of the urine samples, and two phosphate mixtures were prepared with the same total P concentration and of the same pH. Both the urines and the phosphate mixtures were then titrated in a series of stages with the results given in Table IV.

The contrast in buffer action of urine and of a phosphate solution of the same P concentration, especially in the range above pH 7.40, is thus very strikingly brought out. This could not possibly

TABLE III.

Urine sample.	Dog 10.		Dog 11.	
	Buffer value.	Mean pH.	Buffer value.	Mean pH.
5	0.009	6.00	0.028	6.18
	0.012	6.55	0.026	6.68
	0.013	7.10	0.028	7.18
	0.026	7.60	0.032	7.68
6	0.010	5.86	0.046	6.40
	0.010	6.30	0.055	6.90
	0.011	6.80	0.050	7.27
	0.013	7.25	0.056	7.53
	0.015	7.50		
	0.025	7.80		
8	0.014	6.20	0.038	6.14
	0.016	6.65	0.036	6.64
	0.018	7.31	0.046	7.14
	0.023	7.70		

TABLE IV.

pH range of urine.	$\Delta$ pH	Mean pH.	Phosphate concentration.	Cc. 0.1 N NaOH required per 100 cc.	
				Urine.	Phosphate solution.
6.10-6.60	0.5	6.35	0.0165	4.9	4.2
6.60-7.10	0.5	6.85		5.3	3.2
7.10-7.40	0.3	7.25		4.0	2.6
7.40-8.00	0.6	7.70		13.0	3.7
6.30-6.80	0.5	6.55	0.0233	8.2	4.9
6.80-7.40	0.6	7.10		9.7	7.8
7.40-8.00	0.6	7.70		15.8	5.0

be due to the coming into play of the organic acid-salt buffer system, since the organic acids by direct determination were found to have a pK of 4.1 to 4.3, and at pH 7.4 and above these would no

longer be in the free condition. The markedly high buffer value of urine at pH above 7.4 might very well be the cause of the increasing discrepancy between the acidity of urine as determined by our electrometric method (to the pH of 7.4) and by Folin's titration with phenolphthalein (to a pH of about 8.5) noted previously. We are not prepared to offer at present an explanation of these high buffer values for urine except to suggest that this may be associated with its colloidal properties.

In conclusion we wish to consider the question of the indirect method for determining the base economy of the organism. Theoretically, of course, when the sum of free acid of the urine determined directly by titration and of the amount equivalent to that neutralized by the ammonia is subtracted from the total excretion of acid radicles ( $\text{Cl}$ ,  $\text{SO}_4$ ,  $\text{PO}_4$ , and organic acid), the difference should be the equivalent amount of acid combined with fixed base in the urine. In our studies on the mineral metabolism of dogs (still in progress) we measured the elimination of the various basic and acid radicles but were not able to demonstrate the reliability of this assumption. The base actually found by analysis and that calculated as mentioned above may sometimes agree with a fair degree of approximation (3 to 11 per cent) in normal urines, but under experimental conditions the disagreement between these two values becomes very great. The main reason for this undoubtedly is the fact that urines do not titrate like a mixture of the pure substances concerned. Urine acidity determinations, even when made by a reliable electrometric method as detailed in this paper, have a limited significance and should, therefore, be regarded less as strictly quantitative than as indications of the general trend of the metabolism of the organism.

#### SUMMARY.

A simple method is described suitable for titrating electrometrically to a desired pH in the presence of quinhydrone. Urine is titrated to a pH 7.40, the average reaction of the blood, a buffer mixture of the same pH and the titrated urine forming each a half cell, connected by means of an agar-KCl bridge. The end-point of the reaction is registered by the zero deflection of the galvanometer. The significance of the urinary acidity and of the urine buffer value is discussed.

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**PROCEEDINGS OF THE AMERICAN SOCIETY OF  
BIOLOGICAL CHEMISTS.**

**TWENTY-FIRST ANNUAL MEETING.**

**Rochester, New York, April 14-16, 1927.**



## THE COMPOSITION OF THE RESIDUAL NITROGEN FRACTION IN THE URINE.

By ALLAN WINTER ROWE AND BERNARD EMERSON PROCTOR.

*(From the Department of Chemistry, Evans Memorial and Boston University  
School of Medicine, Boston.)*

If the sum of the urea, ammonia, uric acid, and creatinine nitrogen of a given urine be deducted from the total amount, there will remain a small quantity, the so called undetermined or residual nitrogen. In health this usually corresponds to about 6 per cent. Several observers have recorded a relative increase in this quantity with diminution of the total nitrogen elimination which first becomes pronounced when the protein metabolism falls appreciably (about 5 gm. of nitrogen) below the minimum maintenance level. One of us has recorded similar large increments in the relative value of the residual fraction in a variety of disease states even though the protein metabolism is at a level well above the maintenance level. Thyroid and pituitary disease, primary anemias, leucemias, lesions of the central nervous system, malignancy, and syphilis, all show well defined tendencies to influence this quantity.

The present study is concerned with the composition of this residual fraction. Amino acid nitrogen, normally from 1.5 to 3.0 per cent of the total is found to increase to values approaching 6 per cent. Other observers in certain disease conditions have recorded yet higher figures. Creatine nitrogen, usually considered absent from the urines of healthy adults, gives values up to 0.6 per cent. Hippuric acid nitrogen, the normal values for which range from 0.5 to 0.7 per cent, shows but little upward tendency, the controls of this series showing 0.5 per cent, the pathological subjects 0.7 to 0.8 per cent.

The summation of the nitrogen derived from these three sources leaves about 3 per cent of the total of the controls unaccounted for, and from 5 to 8 per cent in the disease group.

As the urine contains several substances in which both nitrogen and sulfur occur, a study was made of the neutral sulfur fraction



to ascertain indirectly whether an increase in these substances could account for the difference. The control group gave an average of 66 mg. of neutral sulfur, while the disease groups ranged from 61 to 78. Patently these factors cannot account *in toto* for the increase in the unexplained residual fraction. It was noted, however, that the neutral sulfur fraction shows a relative increase in disease above the normal level of health.

A tentative conclusion from this phase of the work is that the increase in the residual fraction is seemingly due both to augmentation of substances known to be present in the urine and also to increases which may derive from unrecognized compounds or even the appearance of nitrogen-containing products normally not present in the urine. This question is now under investigation.

#### **A NEW MICRO METHOD FOR THE DETERMINATION OF POTASSIUM IN PHYSIOLOGICAL MATERIAL.**

BY ALFRED T. SHOHL AND HELEN B. BENNETT.

*(From the Department of Pediatrics, Yale University, New Haven.)*

Potassium is precipitated as potassium chloroplatinate according to the usual micro technique. The precipitate is washed in the centrifuge tube with alcohol and ammonium chloride. By the addition of potassium iodide the precipitate is converted to potassium iodoplatinate. Then this is determined either colorimetrically, using potassium chloroplatinate as a standard, or iodometrically with sodium thiosulfate, or by a combination of the two methods. The method is applicable to serum, whole blood, and other physiological material. Although no exhaustive attempt has been made to define the limits of the method, the colorimetric determination is used conveniently for 0.10 to 0.4 mg. of potassium, while titration procedure is applicable to 0.4 mg. or more of potassium.

#### **THE DETERMINATION OF SMALL AMOUNTS OF FATTY ACIDS.**

BY W. R. BLOOR AND R. G. SINCLAIR.

*(From the Department of Biochemistry, the University of Rochester School of Medicine and Dentistry, Rochester, New York.)*

Two methods for this purpose are available at the present time, the nephelometric method and the oxidative method of Bang.

The nephelometric method gives satisfactory results with fatty acid mixtures, such as ordinarily occur in blood, but since the nephelometric values of the solid and liquid acids are quite wide apart, conditions may occur, as for example in alimentary lipemia or other acute lipemia, in which the blood fatty acid mixture may change to such an extent that inaccurate values may be obtained. In Bang's oxidative procedure the values for the different fatty acids and even for cholesterol are fairly close together, a fact which promised a definite advantage in working with unknown fatty acid mixtures. As described by Bang and later by Blix the procedure could not be made to give consistent results. The faults were found to be partly in minor details but mainly in the fact that oxidation was not complete, an end-point being chosen at a point where only about two-thirds of the theoretical oxidation had taken place. For this reason workers with the method do not agree with each other even on the basic values for the fatty acids.

A modification is described in which complete oxidation is brought about and theoretical values obtained for the ordinary fatty acids and cholesterol. The procedure has the advantage of requiring only common laboratory apparatus.

#### A COLORIMETRIC METHOD FOR THE DETERMINATION OF SULFATE IN SERUM.

By ROGER S. HUBBARD.

*(From the Laboratories of the Clifton Springs Sanitarium and Clinic, Clifton Springs, New York.)*

The proteins in serum are precipitated by trichloroacetic acid, and the sulfate precipitated from the protein-free fluid by a solution of benzdine in acetone. The precipitate is then washed with acetone, dissolved in dilute hydrochloric acid, and its benzdine content determined colorimetrically after oxidation with hydrogen peroxide and ferric chloride. A dilute solution of benzdine is used as a standard. Phosphates in greater concentrations than occur in the blood are not precipitated by this procedure, and recovery of sulfates in amounts equivalent to 0.001 to 1.0 mg. of sulfur is possible. Sodium chloride in a concentration twice as great as that of the blood does not interfere with the precipitation. Potassium sulfate added to blood is recovered over a wide

range of concentration, and if the filtrate from the trichloroacetic acid treatment is precipitated with barium chloride the later addition of benzidine dissolved in acetone gives no further precipitate. It is possible to carry out determinations on as little as 2 cc. of serum by this procedure.

#### THE ESTIMATION OF TOTAL SUGAR IN BLOOD AND URINE.

BY M. R. EVERETT AND H. A. SHOEMAKER.

*(From the Department of Biochemistry and Pharmacology, University of Oklahoma Medical School, Norman.)*

Recent experiments have called to our attention certain defects of the Folin and Berglund hydrolysis methods which cause low and irregular values for total sugar, especially with blood filtrates. These disturbances are at least partly due to the presence of rather large amounts of sodium chloride and of a second inhibitory substance introduced by the alkali used for neutralization.

Sodium chloride, in the concentration employed, causes a remarkable fading of the color in the Folin-Wu determination. The decrease may amount to 50 per cent in the course of 25 minutes. Such rapid fading makes accurate reading impossible, even when the same amount of sodium chloride is present in the standard. Other chlorides act similarly and these effects are peculiar to the Folin-Wu and the new Folin methods. The fading is not exhibited by the Sumner method or the latest Benedict method. Equivalent mixtures of hydrochloric acid and sodium hydroxide prove to be even more inhibitory than sodium chloride, the responsible substance being an impurity in the alkali. Silicates are suspected.

Sodium and potassium sulfates have very little effect upon the color production in any of the four methods. The potassium salt has certain advantages and upon this basis we propose the following new method, applicable to all four colorimetric procedures. 8 cc. of blood or urine filtrate are heated on the boiling water bath with 1 cc. of sulfuric acid solution in a specially designed 10 cc. volumetric flask. After cooling and neutralizing the mixture with 1 cc. of a special, equivalent potassium hydroxide solution, the desired analytical method is applied. Standards similarly diluted with acid and alkali are used. At present we are employ-

ing a heating period of 75 minutes and 2.6 N acid and alkali. The new method combines the desired features of careful neutralization, strictly comparative procedure, minimum inhibition of color production, and prevention of sugar destruction.

Analytical data are presented to show various glucose equivalents, the effects of hydrolysis on known sugars, blood, and urine, salt effects, etc.

#### **A METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF COPPER IN PROTEIN AND OTHER ORGANIC MATERIALS.**

By BYRON M. HENDRIX.

*(From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston.)*

A weighed amount of material upon which a copper determination is to be made is ashed in a muffle furnace at as low a temperature as possible. The ash is dissolved in sulfuric acid. A small amount of tartaric acid is then added and the solution made alkaline with sodium hydroxide. Then the solution is diluted to such a volume that 2 cc. of it will contain at least 0.1 mg. of Cu. 2 cc. of the solution are transferred to a Folin-Wu sugar tube; 2 cc. of an alkaline tartrate solution (made by dissolving 40 gm. of anhydrous sodium carbonate and 7.5 gm. of tartaric acid in water and diluting to 1 liter) and about 50 mg. of symmetrical diisopropyl hydrazine hydrochloride are added. The tube is then heated in a boiling water bath. After 6 minutes, the tube is removed from the boiling water bath and cooled under the tap. 2 cc. of Folin-Wu phosphotungstic-phosphomolybdic acid solution are added. After 2 minutes, the solution is diluted to 25 cc. and allowed to stand for 5 minutes *but not much longer*. The solution is now compared in a colorimeter with a standard solution of copper which has been treated at the same time in exactly the same way as the unknown.

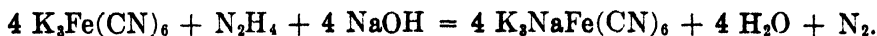
If the material to be analyzed contains iron or any other easily reducible metal, this must be removed before the copper determination can be made.

## GASOMETRIC DETERMINATION OF BLOOD SUGAR.

BY DONALD D. VAN SLYKE AND JAMES A. HAWKINS.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

The method depends on the reduction of ferricyanide by the sugar, and the determination of the excess ferricyanide, by measuring the nitrogen gas evolved by the reaction with hydrazine:



The reagent solution contains 8 gm. of potassium ferricyanide, 75 gm. of  $\text{K}_2\text{CO}_3$ , and 75 gm. of  $\text{KHCO}_3$  per liter. 1 volume of this is mixed in a tube of 16 mm. diameter with 2 volumes of Folin-Wu blood filtrate, containing 0.04 to 0.35 mg. of glucose per cc. The mixture is heated 15 minutes by immersion in boiling water. The solution is cooled in water to room temperature, and is re-saturated with air by stoppering and shaking the tube 1 minute. 2 cc. of a solution, prepared by mixing 1 volume of saturated hydrazine sulfate solution with 1 volume of 40 per cent sodium hydroxide, are placed in the chamber of the Van Slyke-Neill<sup>1</sup> manometric blood gas apparatus. 3 cc. of the sugar-cyanide solution are then measured into the chamber from a rubber-tipped pipette (described by Van Slyke and Neill). The reaction of ferricyanide and hydrazine is practically instantaneous. The chamber is evacuated, the mixture is shaken 1 minute, the gas volume is reduced to 0.5 cc., and the pressure in the manometer is read as  $p_1$ . A control without glucose is run, and the pressure read as  $p_0$ . The decrease in pressure,  $p_0 - p_1$ , due to reduction of ferricyanide by sugar, is the measure of the glucose. One control serves for an entire series of sugar determinations, which can be run off at the rate of about 1 each 3 minutes. 1 mg. of sugar causes a drop in the pressure reading of 315 mm. at 15°, 321 at 20°, 326 at 25°, 331 at 30°. The factor per mg. of sugar is constant, within 1 per cent, for amounts of sugar varying within the limits given above for use with the method.

<sup>1</sup> Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.

## THE INORGANIC COMPOSITION OF BONE.

## I. METHODS.

BY M. J. SHEAR AND BENJAMIN KRAMER.

(From the Harry Caplin Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, New York.)

A correct conception of the physicochemical mechanism responsible for bone formation requires a knowledge of the composition of the bone salts as they are first deposited. There are a number of considerations which lead us to believe that the composition of freshly deposited inorganic material of bone is different from that obtained by analyses of older bones. Kramer and Howland recently described improved methods for bone analyses in which 250 to 500 mg. are required for each sample. From their analyses they conclude that the ratio,  $\frac{\text{residual Ca}}{\text{P}}$ , is  $1.94 \pm 0.14$  when the error of each determination is  $\pm 3$  per cent. This indicates the presence of  $\text{Ca}_3(\text{PO}_4)_2$ . We have attempted to determine whether this ratio holds true for bone cells freshly deposited, as, for example, in the provisional zone of calcification *in vivo* and *in vitro*. Inasmuch as the amount of newly deposited bone obtainable for analysis is quite small, we have found it necessary to modify these methods so that analyses of minute quantities might be made. With the procedure outlined here the determination of carbonate may be performed on about 15 mg. This has enabled us to analyze minute specimens, such as the head bones of a 3 months old human fetus in which the total weight of the dry head bones was 17.7 mg. The results of our analyses will be published in a subsequent communication.

The carbonate is determined in Van Slyke's manometric blood gas apparatus. Calcium and phosphorus are determined on aliquots of a 10 cc. solution made by extracting about 5 mg. of bone powder with HCl, using the Kramer-Tisdall method for calcium and the Briggs-Bell-Doisy method for phosphorus. The reproducibility of the  $\text{CO}_2$  method is  $\pm 3$  per cent. Analyses of twelve different specimens of normally and pathologically calcified tissues gave values of  $1.93 \pm 0.07$  for  $\frac{\text{residual Ca}}{\text{P}}$ . The theoretical value of this ratio for  $\text{Ca}_3(\text{PO}_4)_2$  is 1.94.

**THE COLORIMETRIC ESTIMATION OF TYROSINE IN PROTEIN.**

By MILTON T. HANKE.

(From the Otho S. A. Sprague Memorial Institute and Department of Pathology, University of Chicago, Chicago.)

The tyrosine content of proteins has recently been estimated by two processes; namely, that of Folin and Looney, and that of Hanke. The Folin and Looney process invariably gives values that are much higher than those obtained by my process. I have, therefore, carefully compared the two processes on crystallized egg albumin and casein with the following results.

1. Hydrolysis of 2.4734 gm. of egg albumin for 24 hours with  $\text{H}_2\text{SO}_4$  (15 cc. of 95 per cent  $\text{H}_2\text{SO}_4$  + 100 cc. of  $\text{H}_2\text{O}$ ) destroys tryptophane completely. The hydrolysate of 3.0000 gm. of casein contains a trace of tryptophane.

2. The Folin and Looney process indicates the presence of 104 mg. of tyrosine (4.2 per cent) in egg albumin and 167 mg. (5.56 per cent) in casein. This agrees almost perfectly with the values determined by Folin and Looney on the alkaline hydrolysates of these proteins.

3. Precipitation with  $\text{Ag}_2\text{SO}_4$  and baryta divides the material into two fractions, a silver precipitate which contains all of the histidine and none of the tyrosine and a silver filtrate which contains all of the tyrosine. The silver precipitate does not give a Millon reaction and previous experiments have shown that it does not contain tyrosine; but it gives a blue color with the Folin and Looney reagent equivalent to 16.45 mg. of tyrosine in the case of egg albumin and 19.2 mg. in the case of casein.

4. The silver filtrate is boiled with mercuric acetate. The cooled liquid is treated with  $\text{NaCl}$ . A white or gray precipitate is obtained that contains all of the tyrosine contaminated with a small amount of other amino acids.

5. The Hg precipitate from egg albumin contains 49.37 mg. of tyrosine (2 per cent) by the Hanke and Koessler method, which is characteristic for tyrosine, and 55.60 mg. (2.25 per cent) by the Folin and Looney method. Casein yields 135 mg. (4.5 per cent) of tyrosine by either method. Tyrosine that is added to this material is indicated quantitatively by either method.

6. The filtrate should not contain tyrosine. A Millon reaction

carried out directly on this liquid is negative. The liquid is evaporated, the dry residue extracted with concentrated HCl, and the HCl removed by evaporation. The concentrated material does not give a Millon reaction. A positive reaction is obtained when 0.1 mg. of tyrosine is added. The Hg filtrate does not contain tyrosine. The Folin and Looney process, nevertheless, indicates the presence of 10.50 mg. of tyrosine in the case of egg albumin.

7. Some of the substance that gives a color with the Folin and Looney reagent is lost. The loss is certainly not mechanical.

#### CONCLUSIONS.

The Folin and Looney process is a test for reducing substances. Beside tyrosine, the two proteins studied contain at least one other reducing substance which I shall call the X factor. X factor reduces  $\text{Ag}_2\text{O}$  at  $0^\circ\text{C}$ . and  $\text{Hg}(\text{OAc})_2$  at  $100^\circ\text{C}$ . It is, therefore, a stronger reducing agent than tyrosine and some of it is lost during the process just outlined. Considerable of the X factor is precipitated by Ag in alkaline solution, a small amount is carried down by the Hg precipitate, and some appears in the Hg filtrate. The Folin and Looney process gives an erroneous conception of the tyrosine content of the two proteins studied and the values obtained for other proteins may be similarly erroneous.

#### THE REDUCTION OF CYSTINE-HYDROGEN PEROXIDE AS A CATALYST.

By JAMES C. ANDREWS.

*(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)*

The effect of hydrogen peroxide in effecting a true electronic equilibrium between cystine and cysteine reported by Kendall and Nord<sup>2</sup> led to attempts to reduce cystine completely to cysteine by the use of lower voltages than those previously found necessary by the writer (3 to 5 volts) in presence of small amounts of hydrogen peroxide. If the cathode liquid is allowed to become alkaline a potential of 3 volts will effect complete reduction although several days electrolysis is required. This might be

<sup>2</sup> Kendall, E. C., and Nord, F. F., *J. Biol. Chem.*, 1926, lxi, 295.



ascribed to the presence of small amounts of sodium disulfide resulting from slight decomposition of the cystine. Addition of  $\text{H}_2\text{O}_2$  caused no change in results. In acid solution a complete reduction was not obtained with the above voltages either in presence or in absence of  $\text{H}_2\text{O}_2$ .

Measurement of decomposition voltages of cystine solutions under varying conditions of pH, with and without  $\text{H}_2\text{O}_2$  present, showed no effect on the part of the  $\text{H}_2\text{O}_2$ . The critical decomposition voltage (about 3 to 3.5 volts) is evidently not altered by the presence of  $\text{H}_2\text{O}_2$ .

While these results do not disprove the formation of such a compound of hydrogen peroxide and cystine as Kendall and Nord describe they seem to indicate that it fails to function, both in initiating the electrolytic reduction of cystine and in bringing that reduction to a quantitative conclusion.

### THE RESOLUTION OF RACEMIC CYSTINE.

By JAMES C. ANDREWS.

*(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)*

Gortner and Hoffman<sup>3</sup> have recently reported the failure of several attempts to resolve racemic cystine and have therefore concluded that "it may not be racemic cystine but rather that it may be an internally compensated form." However, their conclusion is at least partially incorrect, as indicated by the following.

A large sample of inactive cystine, dissolved in the smallest possible amount of HCl, was fractionally reprecipitated by successive addition of small portions of alkali. The fractions were washed electrolyte-free, dried, and their rotations taken. From these samples a series of  $[\alpha]_D$  values was obtained which varied from  $-5.7^\circ$  to  $+3.0^\circ$ . The variation was irregular and indicated alternate supersaturation with respect to the *d* and *l* phases. Of seven fractions obtained from one inactive sample, only one fraction was completely inactive. The above results would not have been possible had the inactive cystine been entirely in an internally compensated form. They do not, however, negate the presence of a certain proportion of mesocystine.

<sup>3</sup> Gortner, R. A., and Hoffman, W. F., *J. Biol. Chem.*, 1927, lxxii, 433.

THE OPTICAL ACTIVITY OF *L*-CYSTINE.

BY JAMES C. ANDREWS.

(From the Department of Physiological Chemistry, School of Medicine,  
University of Pennsylvania, Philadelphia.)

It has been stated that *l*-cystine as ordinarily prepared from keratins cannot be identical in configuration with cystine in protein combination because of partial racemization during the hydrolysis of the protein. However, the evidence points to the  $[\alpha]_D^{25}$  value of  $-215^\circ$  as being its maximum rotation for the following reasons.

1. Because of solubility differences, the optical activity of partially racemized samples ( $[\alpha]_D = -100^\circ$  to  $-200^\circ$ , for example) may be continuously raised by water extraction. Water extraction of samples with values of over  $-210^\circ$  has little or no effect, indicating absence of the more soluble phase.

2. Hydrolyses of hair with HCl at lower temperatures have not yielded cystine samples of any higher activity.

3. After removing all cystine possible from a hair hydrolysate by isoelectric precipitation, a further fraction may be obtained by addition of alcohol. This second fraction always shows a much lower optical activity than the first. The higher solubility of the racemic form permits its escape into the filtrate from the isoelectric precipitation and thus makes possible an isoelectric precipitate of maximum activity, even if some racemization has occurred.

4. Rapid isoelectric precipitation produces a greater proportion of the *l* form than even the relative solubilities would require because of a tendency on the part of the *dl* form to supersaturate. A partially racemized sample, on crystallizing out of solution, will deposit the pure *l*-cystine (hexagonal plates) first of all but as the crystals stand in contact with the mother liquor, some replacement by the *dl* form takes place resulting in rosettes of *dl* needles with the whole rosette of definitely hexagonal shape.

A value of  $-252.2^\circ$  for  $[\alpha]_D$  of cystine was reported by Rothera<sup>4</sup> who does not, however, sufficiently define the conditions employed to make comparisons possible. A recent paper by Gortner and Hoffman<sup>3</sup> reports a value of  $-242.6^\circ$  which these writers believe

<sup>4</sup> Rothera, C. H., *J. Physiol.*, 1905, xxxii, 179.

to be the highest rotation for cystine on record. However, when corrected for temperature and concentration of acid this sample does not appear to have any higher activity than those reported by the present writer. Gortner and Hoffman's figure was obtained in a 1 per cent solution of the sample in approximately 0.1 N HCl at 20°C. However, a comparison can only be made by extrapolating on our curve of optical activity *versus* normality of HCl since we have never succeeded in preparing a 1 per cent solution of cystine in acid as dilute as 0.1 N.

#### THE COLORIMETRIC ESTIMATION OF CYSTINE IN CASEIN BY MEANS OF THE NAPHTHOQUINONE CYSTEINE REACTION.

By M. X. SULLIVAN.

*(From the Division of Chemistry, the Hygienic Laboratory, United States Public Health Service, Washington.)*

The casein hydrolysate (6 hours hydrolyzed with 20 per cent HCl) diluted and decolorized is brought to slight acidity, pH 2 to 3, and made to definite volume with 0.1 N HCl. To 5 cc. of the solution add (a) 2 cc. of 5 per cent NaCN, wait 10 minutes, add (b) 1 cc. of 0.5 per cent 1, 2-naphthoquinone-4-sodium sulfonate, mix, add (c) 5 cc. of 10 per cent Na<sub>2</sub>SO<sub>3</sub> in 0.5 N NaOH, mix, let stand 10 minutes, add (d) 2 cc. of 25 per cent NaOH, mix, add (e) 1 cc. of 2 per cent Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> in 0.5 N NaOH. Compare with standard (50 mg. of cystine similarly hydrolyzed, treated with norit, neutralized, and diluted).

In other experiments hydrolysis was done in the presence of reducing agents such as SnCl<sub>2</sub> and TiCl<sub>3</sub>, with little if any humin formation. The hydrolysates, freed from Sn and Ti without use of norit, were analyzed for cystine, as given above, with unheated cystine as standard. In both procedures the percentage of cystine in casein was found to be considerably higher than generally accepted. Consideration is being given to the possibility that in the casein as such or in the hydrolysates there are other substances which react in the naphthoquinone test as cystine does.

**THE NORMAL URINARY PIGMENT. A NEW METHOD FOR ITS EXTRACTION.**

By DAVID L. DRABKIN.

(From the Department of Physiological Chemistry, University of Pennsylvania, School of Medicine, Philadelphia.)

Normal butyl alcohol has been found to be an excellent solvent for the extraction of the normal urinary pigment (urochrome). A rapid extraction by shaking at room temperature has proved best. It is most important to control the hydrogen ion concentration by acidifying the urine with acetic acid. Above pH 4.5 butyl alcohol extracts practically no pigment. At this pH extraction begins and approaches a maximum at pH 3.9. As much as 81 per cent of the coloring matter could be removed by successive extractions, under proper conditions.

Only the mildest procedures were used in the further purification of the butyl alcohol extract. These consisted mainly of washing with 4 volumes of water, concentrating to a small volume *in vacuo*, and repeatedly extracting with chloroform, benzene, amyl acetate, amyl alcohol, and ether. A reddish brown, gummy product was obtained. It was free from uric acid, urea, urobilin, and hematoporphyrin, was soluble in water and, when sufficiently diluted, reproduced the original urinary color.

The concentrated, aqueous solution of the purified product differed from the urochromes hitherto described in that it was not precipitated from solution by the so called alkaloidal reagents. Complete precipitation was obtained however, by means of various salts of Ag, Ba, Cu, Fe, Hg, Pb, and Zn. With increasing acidity, at pH 6.8 butyl alcohol suddenly extracts most of the pigment from its aqueous solution, while at the same pH, with increasing alkalinity, the pigment just begins to pass from the butyl alcohol into the water. At pH 5.4, the aqueous layer is completely decolorized by butyl alcohol and at pH 8.0 nearly all the coloring matter shifts back to the aqueous layer. Complete decolorization of the pigment solution occurred after treatment with zinc dust and hydrochloric acid and color was restored with hydrogen peroxide—manifestations, probably, of reduction and oxidation. The biuret test was negative. Qualitative tests for sulfur have, thus far, proved unsatisfactory. A very faint Adam-

kiewicz reaction was obtained and a positive Millon test. Solutions of the pigment absorbed light uniformly in the violet and ultra-violet, complete absorption beginning at 4250Å. The fluorescence of the pigment between 3680Å. and 3122Å. was brought out by a Wratten No. 18 filter and a quartz mercury arc lamp.

By means of a solution containing a weighed quantity of dried, purified pigment the previously used empirical standard<sup>5</sup> has been evaluated in terms of mg. of pigment. 1 unit of pigment was found to be equivalent to 3.82 mg. of pigment. The average figure for the 24 hour pigment output of an adult male was calculated as 72.6 mg. Dombrowski's<sup>6</sup> figures are 6 to 10 times higher.

#### VARIATIONS IN THE URINARY AND BLOOD ACETONE BODIES DURING THE DAY IN CHILDREN ON KETOGENIC DIETS.

BY IRVINE McQUARRIE AND H. M. KEITH.

(From the Department of Pediatrics, the University of Rochester, Rochester, New York.)

The observation that certain children with epilepsy, while on a ketogenic diet, had occasional convulsive seizures, which occurred only in the early morning hours, suggested the need for information regarding the variations in the urinary and blood acetone bodies during the day under these conditions. Repeated observations were made on one epileptic subject and on each of four normal children, ranging in age from 11 to 13 years. Urine was collected successively in 2½ hour periods, while the subjects remained in bed on a diet consisting of 32 gm. of protein, 200 gm. of fat, and 28 gm. of carbohydrate, divided into either three or four equal meals, 5 hours apart. For different periods of the day, wide variations were noted both in the level of the blood acetone bodies and in the rates of their excretion by way of the urine. Practically the same type of curve was found to represent the excretion in all cases in which the diet was taken. This showed the lowest rate to occur during the early morning hours from 3 a.m. to 8 a.m., and the highest in the afternoon between 2.30 and 5 p.m. Where a 10 p.m. meal was given in addition to the three meals of the day, a second elevation in the curve usually occurred just after mid-

<sup>5</sup> Drabkin, D. L., *J. Biol. Chem.*, 1926, lxxvii, p. xl.

<sup>6</sup> Dombrowski, S., *Z. physiol. Chem.*, 1907-08, liv, 390.

night. The changes in the acetone body content of the blood were found to parallel those of the urine, being lowest in the morning and highest in the late afternoon. When the degree of ketosis was allowed to fall below a certain level in the case of the only epileptic subject studied so far, convulsive seizures were observed to occur coincidentally with the periods of lowest acetone body excretion. The level of the blood acetone bodies determined soon after a seizure was found on several occasions to be considerably below the average for the same day. Further investigations on epileptic children are being made.

#### **A PREVIOUSLY UNDETECTED COMPONENT OF BLOOD.**

BY ELBERT W. ROCKWOOD AND ROBERT G. TURNER.

*(From the Department of Toxicology, State University of Iowa, Iowa City.)*

Acid hydrolysis of non-protein nitrogen blood filtrate liberates a substance which gives a blue color with the arsenophosphotungstic acid-uric acid reagent. It is carried down by silver lactate but is not freed by NaCl; subsequent treatment by NaCN liberates it. Provisionally it has been named Z. It is not the same as thiasine or substance X of Bulmer, Hunter, and Eagles. It is found in the blood of dogs and man as well as in tissues of kidney, liver, and muscle. A method for its quantitative determination has been developed and our present results indicate that it varies from 0.1 to 1.0 mg. per 100 cc. of normal human blood. There is an apparent increase in leucemia and pernicious anemia, also in diabetes.

#### **THE BEHAVIOR OF INSULIN PREPARATIONS TOWARDS THE URIC ACID REAGENT OF FOLIN AND DENIS.**

BY VINCENT DU VIGNEAUD.

*(From the Department of Vital Economics, the University of Rochester, Rochester, New York.)*

The cystine equivalents of the various fractions arising from Abel's method of purifying insulin were determined by Hunter's modification of the Folin-Looney method. The tests were applied to both the hydrolyzed and unhydrolyzed preparations. The values obtained with the former were higher than with the latter.

The intensity of the reactions paralleled the potency of the fractions quite closely. The results were encouraging as a possible chemical method of assay. It would be admittedly unsuited for very impure samples containing large amounts of protein impurities.

The sulfur of the acid-hydrolyzed insulin was found to be comparatively stable, in contrast to that of the original insulin. This clearly demonstrates the presence of a sulfur-containing moiety which can be broken off by acid hydrolysis, the sulfur of which is comparatively stable towards alkali. The change of stability must be due to the effect of other groups linked to the sulfur-containing portion, similar to the influence which the linking of other amino acids has upon the lability of the sulfur of cystine.

The cystine equivalent of a very purified preparation was found to be 13 per cent by the Folin-Looney method, hydrolyzed with acid. This same preparation assayed only 2 per cent after the labile sulfur had been split out by heating with 0.1 N sodium carbonate for 45 minutes and subsequent acidification and hydrolysis. The Sullivan method for cystine was also used in a similar study on this preparation. The cystine value obtained by this method dropped from 7 per cent to less than 1 per cent. This indicates most strongly that the labile sulfur of insulin originates from some derivative of cystine. The reason for the difference between the cystine equivalent found by the Folin-Looney method and that by the Sullivan method has not yet been ascertained. However, cystine heated for 4 hours with 20 per cent HCl on a boiling water bath, similar to the treatment the insulin had undergone during hydrolysis, gave theoretical values by both methods.

#### SEASONAL PERIODICITY IN MAN.

##### I. A STUDY OF THE BLOOD CHEMISTRY OF NORMAL INDIVIDUALS OVER A PERIOD OF TWO YEARS.

By GEORGE W. PUCHER.

*(From the Department of Biochemistry, University of Buffalo Medical School,  
and the Department of Laboratories, Buffalo General  
Hospital, Buffalo.)*

The data presented in this abstract are a part of the work begun 2 years ago in collaboration with Dr. F. Griffith. The complete

chemical study will comprise both urine and blood analyses at weekly intervals on men and women. The data in this abstract summarize only the blood chemistry of the individuals studied. All the data were collected on fasting subjects that had been in complete rest for at least 30 minutes, before withdrawal of the samples. The conclusions outlined below are obtained by the construction of composite curves representing the averages of all the data collected on the blood over the entire experimental period.

Our study indicates that there are small but definite changes in the blood chemistry of normal men and women at different times of the year. The most definite of these changes are summarized as follows:

(1) The blood sugar shows a definite rise during the summer months and falls again in the winter months. (2) The chlorides of the whole blood drop during the early spring and summer months and rise slightly in the fall. The changes are not as marked as in the case of the blood sugar. (3) The corpuscular volume is somewhat lower during the summer months than in the winter months. (4) Uric acid falls slightly from January to May, rises from May to September and then falls again. The changes although small are regular and beyond the experimental error of the method used. (5) The non-protein nitrogen is low during the summer months rising again in the fall and winter. (6) The blood cholesterol shows a steady rise from January to August and then decreases again. (7) The blood phosphorus is decidedly higher during the summer months and falls during the winter months.

## STUDIES OF THE BLOOD BY VIVIDIALYSIS.

### I. THE BLOOD SUGAR.

BY MARSCHELLE H. POWER AND CARL H. GREENE.

*(From the Division of Medicine, Mayo Clinic and The Mayo Foundation, Rochester, Minnesota.)*

The rotatory and reducing powers of blood dialysates obtained by the vividiffusion technique of Abel and Rowntree have been studied. Normal dogs were operated on under a local anesthetic. Heparin was used as an anticoagulant.

Dialysis was carried out in duplicate, against sodium chloride



solution containing no glucose and against 0.20 per cent glucose solution, respectively. The progress toward equilibrium was followed by determining the reducing powers of simultaneous samples of whole blood, blood plasma, and each dialysate, by both the Folin-Wu and the Shaffer-Hartmann (Somogyi modification, 1926) methods. Equilibrium was established at, or near, the plasma sugar level, in each dialysate. This indicates that relatively little redistribution of sugar takes place between plasma and corpuscles during the drawing and rapid centrifuging of heparinized blood for the separation of the plasma. The distribution found under the conditions of the experiments was approximately 40 per cent in the corpuscles and 60 per cent in the plasma, the latter varying from 0.125 to 0.140 per cent. Since the concentration of glucose in the dialysates also varied between these values, it was possible to obtain more accurate polariscopic readings than in the case of dialysates obtained by *in vitro* methods.

The optical rotatory power was determined in tubes 2 dm. in length, by means of a Schmidt and Haensch polariscope reading to  $0.01^\circ$ , with the green mercury line as a light source.

In general, in those experiments that were continued until equilibrium was attained (6 hours), the rotatory powers of dialysates have been 15 to 30 per cent lower than the reducing powers. In shorter experiments (1 to 4 hours) the rotatory and reducing powers have been more nearly equal. In nine of ten experiments the reducing and rotatory powers have remained constant over long periods, while in only one experiment was the increase in rotatory power described by Lundsgaard and Holbøll, encountered. The composition of the dialysates, now being more completely studied, will obviously help to explain these differences.

#### STUDIES ON BLOOD CREATININE.

By OLIVER HENRY GAEBLER AND ANNA K. KELTCH.

(From the Biochemical Laboratory, State University of Iowa, Iowa City.)

Addition of phosphotungstic acid to the picric acid filtrate of bloods containing as much potassium as human blood causes a precipitate of potassium "picrophosphotungstate," which adsorbs creatinine. The separated precipitate decomposes on treatment with alcohol-ether mixture, yielding potassium picrate and

an alcohol-ether solution containing phosphotungstic and picric acids and the creatinine originally adsorbed. On adding excess of picric acid to this solution and cooling, the creatinine is precipitated out, and is obtained from this precipitate as creatinine potassium picrate. The presence of even large amounts of creatine introduces no source of error.

The procedure has been applied to blood of dogs with both ureters ligated, or with both kidneys removed, and to blood, pleural transudate, and ascitic fluid, of nephritics. The creatinine potassium picrate obtained was analyzed for creatinine content, for nitrogen (non-picrate) per mg. of creatinine, and was further identified by conversion to creatinine zinc chloride. The amount actually weighed, without corrections, reached 50 per cent of the creatinine content of the blood indicated by the method of Myers.

By modification of the isolation procedure of Behre and Benedict, in which Lloyd's reagent is used, the isolation of creatinine from retention blood has also been accomplished. The procedure is preferable to that given above. Four successive isolations carried out on normal ox blood by this method have yielded a crystalline picrate, precipitating only after addition of potassium, freely soluble in hot picric acid solution, containing 18 per cent of apparent creatinine, and completely removable from its solution in saturated picric acid by kaolin. Further analysis has not been carried out because of the small amount of double picrate available. The amount isolated is only 0.3 to 0.4 mg. per 100 cc. of blood, while the creatinine content of the blood indicated by the usual analysis is 3 to 5 times this amount.

#### **A STUDY OF THE EFFECT OF CREATINE ON GROWTH AND ITS DISTRIBUTION IN THE TISSUES OF NORMAL RATS.**

By ALFRED CHANUTIN.

*(From the Laboratories of Physiological Chemistry, University of Illinois, Urbana, and University of Virginia, University.)*

In view of the possibility that creatine might act as an anabolite and thus be instrumental in promoting an increase in body weight, experiments were conducted on white rats to test this hypothesis. At definite intervals these animals were killed and the tissues were analyzed for creatine in order to determine the extent of

creatine storage. Any significant increase in the creatine content of the tissues of creatine-fed animals would indicate either a creatine reservoir or would reveal a site important in the metabolism of this substance.

Young rats were fed on adequate diets containing creatine (0.67 and 2.67 per cent) for a period of 2 months. The growth curves of these animals did not differ from those of the control animals.

The average creatine concentrations for the tissues of albino rats are as follows: muscle 0.449 per cent, testes 0.281, heart 0.174, brain 0.129, kidney 0.046, liver 0.033, and blood 0.017 (total creatinine).

The liver is the only organ of those enumerated in which a significant increase in creatine concentration was noted in animals fed on creatine-containing diets. There is no evidence of a creatine reservoir in any of the tissues studied. It is suggested that the liver plays an important rôle in the metabolism of creatine.

#### THE "INORGANIC PHOSPHATE" OF MUSCLE.

BY CYRUS H. FISKE AND YELLAPRAGADA SUBBAROW.

*(From the Biochemical Laboratory, Harvard Medical School, Boston.)*

Only a small fraction of what has been regarded as inorganic phosphate in voluntary muscle is actually inorganic. The bulk of it is an unstable compound of creatine and phosphoric acid, which is hydrolyzed on stimulation and resynthesized when the muscle is permitted to recover.<sup>7</sup>

#### OBSERVATIONS ON THE CHEMICAL COMPOSITION OF SYNOVIAL FLUID.

BY F. A. CAJORI AND RALPH PEMBERTON.

*(From the Laboratory of Clinical Chemistry, Presbyterian Hospital, Philadelphia.)*

Synovial fluid and blood plasma have been compared with respect to non-protein nitrogen, urea, and amino acids. Almost identical values were found. The concentration of other diffu-

<sup>7</sup> Fiske, C. H., and Subbarow, Y., *Science*, 1927, lxx, 401.

sible constituents of synovial fluid and blood has previously been shown to be similar.<sup>8</sup>

The proteins of synovial fluid, which are present in lower concentration than in plasma, have been fractionated. The globulin content was found to vary more in different synovial fluids than in plasma and the albumin-globulin ratio is somewhat higher than is usually encountered in plasma.

Synovial fluids with high leucocyte counts are usually acid and contain but traces of sugar. On the addition of glucose to a sterile synovial fluid with a white cell count of 14,000 and pH of 6.50, rapid glycolysis was found to take place. No glycolysis occurred in a sample of the same fluid from which the cells had been removed. The high acidity and absence of glucose characteristic of septic joint fluids are probably ascribable to glycolysis by the leucocytes within the joint cavity.

#### THE MECHANISM OF GASTRIC SECRETION.

##### PRELIMINARY REPORT ON A TECHNIQUE FOR COLLECTING GASTRIC JUICE OF CONSTANT AND REPRODUCIBLE pH.

By FRANKLIN HOLLANDER.\*

*(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)*

By means of the quinhydrone electrode, pH determinations were made on samples of postprandial gastric juice from Pavlov pouch dogs. These samples were obtained by means of a rubber catheter retained in the pouch throughout an entire experiment, the collecting vessel being changed periodically. As reported by other investigators, using titrimetric methods, the larger the sample collected in a given time interval, the higher its acidity—or the lower its pH. Under these conditions, the pH was rarely less than 1.08 (the pH of 0.1 N HCl) and almost never less than 1.00.

<sup>8</sup> Cajori, F. A., Crouter, C. Y., and Pemberton, R., *Arch. Int. Med.*, 1926, xxxvii, 92.

\* Medical Fellow of the National Research Council in Physiological Chemistry.

However, when the juice was collected in such a way as to eliminate prolonged irritation of the gastric mucosa by the rubber catheter, the pH was invariably less than 1.00, provided the volumes were not too small. In fact, most of the samples so collected—more than 2 dozen from four dogs—had a minimum value of  $0.92 \pm 0.01$ . The irregular variation of the few remaining ones can be ascribed to mucus or other acid-neutralizing substances present for various reasons. The new technique now being developed for collecting juice takes advantage of a sphincter-like action at the mouth of the pouch, by reason of which the fluid retained within the pouch can be removed by the rapid insertion and immediate withdrawal of the catheter.

An independent verification of the constancy of this minimum value was obtained in the following way: Graded amounts of histamine were injected subcutaneously, the catheter being inserted in the pouch throughout the experiment—as in the continuous collection method. A small amount of this chemical stimulant resulted in the same parallel relation of volume and pH as that observed in the postprandial series. With relatively large amounts of the histamine, however, the pH fell to the minimum value of  $0.92 \pm 0.01$ —more or less rapidly, depending on the quantity of the drug—and remained there until the rate of flow had diminished significantly. As many as five 15 minute samples possessing this constant pH have been obtained in the course of one experiment.

#### **THE ORGANIC CHLORIDES OF TISSUES AND THEIR POSSIBLE RELATION TO GASTRIC HYDROCHLORIC ACID FORMATION.**

BY MARTIN E. HANKE AND PAUL B. DONOVAN.

*(From the Department of Physiological Chemistry, University of Chicago, Chicago.)*

The problem of the chemistry of gastric hydrochloric acid formation is in a very unsatisfactory state. The subject has been treated very largely from the theoretical point of view, and such experiments as we have are very inconclusive. Moreover, the current theories involve the unreasonable assumption of the separation of the acid locally from an equivalent amount of alkali. Finally the generally accepted theory of Maly would have us believe that a local increased concentration of carbonates and phos-

phates assists in the process of hydrochloric acid formation, notwithstanding the very effective buffer action of these salts of weak acids.

It seemed reasonable, therefore, to develop a theory of gastric acidity production which would not contain this assumption of local alkali formation, and which, in general, would be more amenable to experimental attack. A chemical reaction which seems particularly probable in this connection is the hydrolysis of an alkyl halide, with the production of hydrochloric acid and the corresponding alkyl alcohol  $\text{RCl} + \text{H}_2\text{O} \rightarrow \text{HCl} + \text{ROH}$ . The theory as we have formulated it involves (1) the mobilization of an organic chloride ester in gastric tissue at the time of gastric activity, and (2) the hydrolysis of this chloride ester, possibly under the influence of a specific enzyme activity. The alcohol thus formed, if secreted at all, may be reabsorbed at once, or it may remain in the gastric juice, to be absorbed later in the intestine. (3) The organic chloride is thought to be synthesized at the time of gastric activity, probably in other tissues as well as in gastric tissue, as it is needed, from sodium chloride and the alcohol, with the simultaneous production of alkali  $\text{NaCl} + \text{ROH} \rightarrow \text{RCl} + \text{NaOH}$ , thus accounting for the alkaline tide accompanying gastric secretion. To avoid an apparent contradiction, let it be explained that the alkali production accompanying the synthesis of the ester, though simultaneous with the acidity production, is in no sense coincident with it. The two reactions probably take place at two remote places, and certainly do not occur in the same phase. The feature of this theory is that it permits of the formation of any concentration and amount of hydrochloric acid from *neutral* reactants and with a *neutral* product.

The existence of enzymes in gastric and other tissues specific for the hydrolysis of chloride esters has been demonstrated and already reported on.<sup>9</sup>

The results on the search for organic chlorides in gastric and other tissues may be summarized thus:

1. A standard method for the differential fractional extraction of inorganic (ionized) and organic (unionized) chlorides from tis-

<sup>9</sup> Hanke, M. E., *J. Biol. Chem.*, 1926, lxvii, p. xi.

sues has been developed. The organic chlorides are very soluble in organic solvents, and are associated with compound lipoids.

2. It has been shown that the fraction of organic chloride in some common tissues is very considerable, amounting to 10 to 50 per cent of all the chloride present. The following tabulation gives the distribution of organic chloride in five different tissues.

*Total and Organic Chloride per 100 Gm. of Dry Tissue in Any Case.*

	Total chloride.	Organic chloride.
	gm.	gm.
Fundus.....	0.65-0.75	0.29-0.31
Pylorus.....	0.50-0.65	0.18-0.21
Intestine.....	0.45-0.55	0.14-0.18
Liver.....	0.30-0.35	0.15-0.16
Blood.....	0.90-1.00	0.09-0.13

It seems very significant that the fundus mucosa contains almost twice as much organic chloride per given weight of dry tissue as do the other tissues studied.

3. A preliminary study of some of the chemical properties of the organic chloride has been carried out. In aqueous solution it is hydrolyzed gradually and progressively with the production of both acidity and ionized chloride. The rate of the reaction varies considerably with different preparations; some require 15 minutes at room temperature in 5 per cent solution for complete hydrolysis, others are complete in 1 or 2 minutes. Silver nitrate or sodium hydroxide hastens the reaction, while dilute sulfuric or nitric acid retards it. The highest acidity thus far observed was pH 2.7. The production of acidity and ionized chloride usually runs parallel; occasional discrepancies in some preparations are probably due to an insufficient degree of purity of the material. The best preparations so far obtained contain 6 per cent by weight of chloride, also some phosphorus and nitrogen. The organic chlorides of other tissues appear to have the same chemical properties as those of gastric tissue.

4. A comparative study of the distribution of different types of chloride in the tissues of fed and fasted dogs showed no significant variations. To us this means that in the normal animal there is no appreciable fatigue or exhaustion of the mechanism.

5. These results, it is thought, furnish strong support for the theory that the hydrolysis of organic chlorides plays an important rôle in gastric acidity production.

The presence of such a considerable amount of organic chloride in the mammalian organism (estimated at 15 per cent of the total chloride) probably has great significance in the general metabolism, especially in the regulation of the general acid-base balance. This and similar questions are being investigated.

#### **CHANGES IN CALCIUM LEVEL OF THE BLOOD FOLLOWING SECTION OF THE SYMPATHETIC OR OF THE SPINAL CORD.**

By ALFRED F. HESS, BENJAMIN N. BERG, AND  
ELIZABETH SHERMAN.

*(From the Department of Pathology, College of Physicians and Surgeons,  
Columbia University, New York.)*

When the splanchnic nerve of a dog is severed below the diaphragm, a slight fall in the calcium content of the serum is brought about. This change is marked when both nerves or the plexus are severed, or when the celiac ganglion is removed. The level of calcium may fall to about 6 mg. per cent, and remain low for a week or 2. Tetany did not develop.

On the other hand, a marked increase in serum calcium may follow section of the spinal cord, the percentage rising as high as 17 mg. within 24 hours. It has not been possible to bring about this elevation constantly by means of operative procedure.

#### **CALCIUM AND PHOSPHORUS METABOLISM IN DAIRY COWS.**

#### **II. THE RELATIVE ASSIMILATION OF CLOVER AND ALFALFA HAYS AND OF RATIONS OF VARYING CALCIUM AND PHOSPHORUS CONTENT.**

By WILLIAM A. TURNER, T. SWANN HARDING, AND  
ARTHUR M. HARTMAN.

*(From the Experiment Station, Bureau of Dairy Industry United States  
Department of Agriculture, Beltsville, Maryland.)*

Two mature cows (a Holstein and a Jersey), yielding 10 to 20 kilos of milk per day, were fed for 4 weeks a ration consisting of a good grain mixture and good quality clover hay following which,



during another 4 weeks, the clover hay of the ration was displaced by a good quality of alfalfa hay. Balances indicated a better assimilation of calcium and phosphorus from alfalfa hay than from clover hay.

Immediately following this, two cows (one the Holstein used previously and the other a Jersey), yielding 10 to 20 kilos of milk per day, were fed for 3 weeks a ration consisting of a grain mixture, of good quality but low in phosphorus content, and a good quality of alfalfa hay. During this period there was at least 2.5 times as much calcium as phosphorus in the ration. Following this, for a period of 3 weeks, the phosphorus in the ration was increased by the addition of sodium phosphate so that the calcium in the ration was only 1.25 times as much as the phosphorus. One cow, the Holstein, showed an improved assimilation of calcium and phosphorus on the higher phosphorus ration. The other cow showed no significant change. Previous nutritional history of the cows may explain the difference in their response to the change in ration. Probably the changes in the proportion of calcium and phosphorus in the ration were not of sufficient magnitude to exert any considerable influence on cows in a good state of nutrition.

In this experiment positive calcium and phosphorus balances were observed with cows yielding from 12 to 19 kilos of milk daily on a ration consisting of a good grain mixture and a good quality of alfalfa hay.

#### **SOME EFFECTS OF ASPHYXIA ON BLOOD CHEMISTRY.**

By J. B. COLLIP.

*(From the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.)*

It was noted in the course of an investigation of the blood chemistry of the fetal calf that the serum calcium and inorganic phosphorus values were very high. Thus in 94 fetal calves the average serum calcium was 14.76 mg. per 100 cc., whereas the serum of seven cows averaged 10.5 mg. and the serum of six calves 11.4 mg. The inorganic phosphorus in 71 specimens of fetal blood averaged 11.5 mg. and in six adult animals 3.9 mg. Six very young calves gave an average value of 6.8 for whole blood inorganic phosphorus. Since the blood of new born calves did not give on

analysis values for calcium and inorganic phosphorus comparable to those obtained on the fetus at approximately full term, it was thought that asphyxia might account for the discrepancy observed. As several minutes elapse between the time that the mother animal is bled out and the fetus is available for examination, the latter must of necessity be asphyxiated. The fetal blood samples were taken directly from the heart which was as a rule still beating.

The effect of asphyxia *per se* in different animals is shown in Table I.

The effect of asphyxia on the fetus was determined by direct experiment. A pregnant bitch near term was placed under morphine-ether anesthesia and the abdomen opened. The uterus contained nine fetuses. Blood was drawn from the hearts of five of these. The circulation to that part of the uterus containing the remaining four was then cut off. After 10 minutes of asphyxia blood samples were taken from these asphyxiated fetuses. The blood serum calcium of the normal fetuses averaged 11.5 mg. with 10.9 and 11.9 mg. as the low and high values observed. The serum calcium of the asphyxiated fetuses averaged 13 mg. with 12.7 and 13.2 mg. as the low and high values occurring. There was an average increase of 1 mg. in the inorganic phosphorus of the serum of the asphyxiated fetuses. The maternal blood serum calcium was 9.2 mg.

TABLE I.

	Serum Ca increase.	Inorganic P serum increase.	Inorganic P blood increase.	pH decrease.
9 rabbits.	3.1		1.4	
2 "				0.35
5 "		2.9		
4 cats.	1.7		1.7	
2 "				0.16
3 calves.	0.9		1.0	
2 "				0.18
6 rats.	2.4			
4 "			1.2	
2 "		3.1		
2 dogs.	1.2		0.4	
1 "		0.5		0.26
3 hens.	1.4		2.4	

Asphyxia causes a definite decrease in the pH (0.2 to 0.4) and in the plasma volume. The plasma loses fluid both to the tissues and to the red cells since both hemoglobin and hematocrit values are as a rule raised after asphyxia. The concentration of the blood might in itself be sufficient to account for the increase in serum calcium and inorganic phosphorus. Also the changing pH would tend to cause an increase in organic phosphorus at the expense of the acid-soluble fraction.

VARIATION IN CHEMICAL COMPOSITION OF HEN'S BLOOD  
ACCOMPANYING EGG PRODUCTION.

By J. S. HUGHES, W. L. LATSHAW, AND B. L. SMITS.

(From the Department of Chemistry and Dairy Husbandry, Kansas State  
Agricultural College, Manhattan.)

Hughes, Titus, and Smits<sup>10</sup> have shown that egg production in the hen is accompanied by a marked increase in the calcium content of the blood. In order to determine the relation of egg production to the other mineral elements in the blood a complete mineral analysis of the blood of laying hens has been made. For this purpose 10 cc. of blood were drawn from each of 40 laying hens. The 400 cc. of blood were pooled and the mineral elements were

Mineral Analysis of Blood Plasma of Laying Hens and Mature Roosters.

Element.	Gm. in 1000 gm. plasma.			
	Laying hens. Plasma composited from 40 hens.		Mature roosters. Plasma composited from 25 roosters.	
	Experiment 1.	Experiment 2.	Experiment 1.	Experiment 2.
Calcium.....	0.241	0.245	0.145	0.150
Magnesium.....	0.045		0.039	
Sodium.....	3.399	3.433	3.555	3.385
Potassium.....	0.272	0.284	0.257	0.252
Total phosphorus.....	0.329	0.343	0.134	0.111
Inorganic phosphorus.....	0.032	0.032	0.035	0.035
Chlorine.....	3.777	3.752	4.114	4.002
Sulfur.....	0.940		0.821	

<sup>10</sup> Hughes, J. S., Titus, R. W., and Smits, B. L., *Science*, 1927, lxxv, 264.

determined on the resulting plasma, by standard macrochemical methods. The results are given in the accompanying table.

**OBSERVATIONS UPON THE COMPOSITION OF BLOOD AND MILK  
OF WOMEN DURING THE DIFFERENT STAGES  
OF LACTATION.**

By ICIE G. MACY, JULIA OUTHUSE, M. LOUISA LONG, MINERVA BROWN, HELEN HUNSCHER, AND B. RAYMOND HOUBLER.

*(From the Nutrition Research Laboratories of the Merrill-Palmer School and the Children's Hospital of Michigan, Detroit.)*

Fasting bloods and 24 hour samples of milk were taken from the same mothers during the colostrum, transitional, and early mature stages of lactation. Great individual variations were found both in quantity and quality of milk, but as lactation advances the percentages of water and milk sugar gradually increase while the specific gravity and percentages of total solids, total ash, and proteins decrease. The fat and chlorine content vary irregularly. Albumin nitrogen, determined separately, was found to be lower than values assumed by estimating it as the difference between casein nitrogen and the total nitrogen. The non-protein nitrogen of the milk ranged between 15 and 20 per cent of the total nitrogen. When certain constituents such as sugar, amino acids, calcium, and phosphorus were found to be high or low in the blood, they were correspondingly high or low in the milk.

**THE APPARENT FIRST DISSOCIATION CONSTANT,  $pK_1'$ , OF CARBONIC ACID, THE ACTIVITY COEFFICIENT OF THE BICARBONATE ION IN SOLUTIONS OF HEMOGLOBIN, METHEMOGLOBIN, CYANHEMOGLOBIN, AND NITRIC OXIDE HEMOGLOBIN AT VARYING IONIC STRENGTHS AND THE EXTENSION OF THE DEBYE-HÜCKEL THEORY OF IONIC INTERACTION TO HEMOGLOBIN, BICARBONATE-SODIUM CHLORIDE SYSTEMS.**

By WILLIAM C. STADIE AND EFFIE ROSS HAWES.

*(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia.)*

1. The apparent first dissociation constant,  $pK_1'$  of carbonic acid was calculated from the electrometric  $p\alpha H$ , and stoichio-

metrical total  $\text{CO}_2$  and  $P_{\text{CO}_2}$  in solutions of varying concentrations of carbon monoxide hemoglobin, reduced hemoglobin, methemoglobin, cyanhemoglobin, and nitric oxide hemoglobin and varying ionic strengths  $= \Gamma$ . When plotted against the  $\sqrt{\Gamma}$  straight lines are obtained; *viz.*,  $\text{pK}_1' = \text{pK}_* - \beta \sqrt{\Gamma}$ .

The total activity coefficient of the bicarbonate ion was calculated from  $\text{pK}_1'$ . In all cases  $\log \gamma_{\text{HCO}_3^-}$  plotted against  $\sqrt{\Gamma}$  gave a straight line similar to the linear relation  $\log \gamma_{\text{HCO}_3^-} = -\beta \sqrt{\Gamma}$  of the limiting equation of the Debye-Hückel theory. When  $\text{Hb} = 0$  the theoretical  $\beta_0 = 0.54$  is obtained. With increasing Hb concentration however  $\beta$  decreases, the extent of decrease varying with the hemoglobin derivative.  $\beta$  can be expressed by the equation  $\beta = 0.54 - \sigma [\text{Hb}]$ .

$\text{pK}_*$ , the value of  $\text{pK}_1'$  at finite hemoglobin concentrations and  $\Gamma = 0$ , also decreases according to the equation  $\text{pK}_* = 6.33 - \rho [\text{Hb}]$ .

$\rho$  and  $\sigma$  are constants characteristic of the derivative of hemoglobin.

If the activity coefficient  $\gamma_{\text{HCO}_3^-}$  is equated to two activity coefficients  $\gamma_*$  and  $\gamma_0$ , they may be calculated by the equations

$$\log \gamma_{\text{HCO}_3^-} = \log \gamma_* + \log \gamma_0$$

$$\log \gamma_* = -\rho [\text{Hb}]$$

$$\log \gamma_0 = -[0.54 - \sigma [\text{Hb}]] \sqrt{\Gamma}$$

These activity coefficients allow the calculation of  $\text{pK}_1'$  over wide ranges of Hb and  $\Gamma$ .  $\gamma_*$  measures the effect of hemoglobin on  $\gamma_{\text{HCO}_3^-}$ , while  $\gamma_0$  gives the effect of salt.

At constant  $\Gamma$ ,  $\text{pK}_1'$  and  $\gamma_{\text{HCO}_3^-}$  are constant in hemoglobin solutions at  $\text{pH}$  values above and below the isoelectric point showing that the activity coefficient of  $\text{HCO}_3^-$  is the same whether combined with sodium or hemoglobin. This indicates  $\text{HbHCO}_3$  to be completely dissociated.

2. The above experimental values of  $\text{pK}_1'$  and  $\gamma_{\text{HCO}_3^-}$  may be calculated theoretically by means of the Debye and Hückel theory of interionic action in solutions when the theory is extended to concentrated hemoglobin solutions.

The extension is discussed in detail and may be summarized as follows: Theoretical and experimental reasons show that the ionic strength valence of the colloidal hemoglobin ion is 1. *b*, the ionic diameter of the  $\text{HCO}_3^-$  ion, was determined. It is approximately  $2 \times 10^{-8}$  cm. Hemoglobin derivatives apparently *increase* the dielectric constant of water proportionally to the concentration whereas most non-electrolytes decrease it. This effect is given by

$$D_{\text{Hb solution}} = D_{\text{H}_2\text{O}} (1 + \delta[\text{concentration of Hb}])$$

$\delta$  is a constant characteristic for *each* derivative of hemoglobin. On the basis of  $\delta$  the derivatives may be roughly divided into two groups

Observed $\delta$	
0.017	Carbon monoxide hemoglobin.
0.016	Nitric oxide hemoglobin.
<hr/>	
0.029	Reduced hemoglobin.
0.032	Methemoglobin.
0.027	Cyanhemoglobin.

It is further shown theoretically that  $\gamma_{\text{HCO}_3^-}$  may be split into two activity coefficients  $\gamma_*$  and  $\gamma_0$ .  $\gamma_*$  measures the effect of hemoglobin on the bicarbonate ion, whereas  $\gamma_0$  measures the effect of salt. Both  $\gamma_*$  and  $\gamma_0$  may be calculated theoretically from the free energy of the bicarbonate ion, hence  $\gamma_{\text{HCO}_3^-}$  and therefore  $\text{pK}_1'$  may be calculated in solutions in which hemoglobin varies from 0 to 20 mM per liter and  $\Gamma$  (the ionic strength) from 0 to 0.5 M.

The equations for these calculations are:

$$\log \gamma_{\text{HCO}_3^-} = \log \gamma_* + \log \gamma_0$$

$$\log \gamma_* = - \frac{N e^2 \delta[\text{Hb}]}{1.6 b D_0 R T} = - \frac{1.6 \cdot 10^{-8} \delta[\text{Hb}]}{b}$$

$$\log \gamma_0 = - \left( \beta_0 - \frac{3}{2} \delta[\text{Hb}] \right) \sqrt{\Gamma}$$

$$\text{pK}_1' = 6.33 + \log \gamma_{\text{HCO}_3^-}$$

Where  $N$  = Avogadro's number =  $6.07 \cdot 10^{23}$ .

$e$  = elementary electronic charge =  $4.77 \cdot 10^{-10}$  electrostatic units.

$D_0$  = dielectric constant of water = 72.5 at 38°C.

$R$  = gas constant.

$T$  = absolute temperature.

$\beta$  = 0.53 calculated from universal constants.

6.33 =  $pK_1'$  at infinite dilution in water.

$\delta$  and  $b$  are given above.

$pK_1'$  calculated agree within the limit of experimental error ( $\pm 0.02$ ) with  $pK_1'$  observed over all ranges of Hb and  $\Gamma$  studied.

Since  $\delta$  is different for each hemoglobin derivative  $pK_1'$  is likewise different. It is possible with the above data to estimate  $pK_1'$  in red cells. For reduced cells  $pK_1' = 5.85$ ; for oxidized cells  $pK_1' = 5.97$ . These calculated values were confirmed by direct determinations in hemolyzed human, horse, and beef red cells.

#### A STUDY OF THE ACID-BASE EQUILIBRIA OF THE BLOOD IN CASES RECEIVING ALKALI.

BY VICTOR C. MYERS AND EDWARD MUNTWYLER.

*(From the Biochemical Laboratory, State University of Iowa, Iowa City.)*

Determinations have been made of the pH (electrometrically and colorimetrically),  $CO_2$  content, plasma and whole blood chloride, serum sodium, and hemoglobin on a series of 80 cases (160 specimens) most of whom were receiving alkali. In about 80 specimens the colorimetric method of estimating the pH was checked against the electrometric method. The agreement between the two methods was reasonably satisfactory. An uncompensated alkalosis was encountered in a considerable number of cases. With the rise in blood bicarbonate there was a definite fall in the plasma chloride, amounting on the average to 30 to 40 mg. calculated as sodium chloride. Uncompensated alkalosis was generally associated with a definite rise in the serum sodium, although high sodium figures were encountered in the absence of alkalosis. In several instances they were apparently dependent upon high plasma chlorides. An effort was made to check the  $CO_2$  and Cl bound by sodium with the figures found for sodium. Cases of uncompensated alkalosis generally showed a considerable excess of sodium. However, in many instances the excess of sodium was difficult to explain. An effort was made to secure information on the "chloride shift" on the basis of the figures obtained for plasma and whole blood chlorides and hemoglobin.

## STUDIES ON THE RATE OF OXYGEN ABSORPTION BY BLOOD.

BY W. S. MCELLROY AND C. C. GUTHRIE.

*(From the Department of Physiology, University of Pittsburgh, Pittsburgh.)*

From observing the slowness with which influenzal pneumonia bloods absorbed oxygen on shaking in air, a method for measuring the rate was devised, search of available literature revealing no description of a satisfactory method of the type desired.<sup>11</sup>

The apparatus consists of an electrically driven shaker and mercury blood-gas pump and analyzer, equipped with a small calibrated manometer. The filling reservoir serves for the absorbing chamber, and to this the manometer is attached. Colored alcohol is used for displacing fluid, and a T-tap controls connections between the chamber, manometer, and outside air.

Blood to be tested, after defibrinating, or treating with an anti-coagulant, usually oxalate, is drawn into the pump through a side tube and the gases are extracted and expelled. It is then run into the absorbing chamber and shaken until the volume of gas in the chamber becomes stationary. Manometer readings are taken at 5 second or longer intervals. Blood gas analyses are made upon part of the blood sample after the partial gas extraction, and after saturating, as desired. Tests are made at room temperature, which averages 22°C. Usually at least duplicate tests are made, and two operators and two sets of apparatus employed. With due regard to stable room temperature, results of satisfactorily uniform accuracy are obtained.

An unsolved problem is satisfactory viscosity evaluation and control. That it is a factor of importance is clear from experience as well as from theoretical considerations. So results obtained from different specimens of blood may be compared only with caution.

The technique employed reduces the oxygen to about 60 to 70 per cent saturation level, and therefore the results are for the upper physiological range.

With due consideration to viscosity and other factors, results obtained under a number of conditions seem to warrant the fol-

<sup>11</sup> Guthrie, C. C., Publications from the University of Pittsburgh School of Medicine, Studies on epidemic influenza, 1919, 155. McEllroy, W. S., and Guthrie, C. C., *Am. J. Physiol.*, 1920, li, 195.



lowing conclusions. (1) Normal blood of man, dog, ox, pig, sheep, and rabbit, becomes saturated from the 70 per cent level in 4 to 6 minutes. (2) Corresponding sera or plasmas saturate in about  $\frac{1}{2}$  minute. (3) Mixtures of blood and serum (or plasma) require less time than blood alone, this varying in proportion to the amount of hemoglobin. (4) Dilution of the blood with other solutions, as 0.9 per cent NaCl, has an effect comparable to that of dilution with serum. (5) Reduction in the amount of available methemoglobin, as in methemoglobinemia, also shortens the time required for saturation. (6) Laking alters the rate, in which changes in viscosity must be considered. Laking by freezing is the most promising method. (7) The addition of acids, alkalies, and neutral salts does not greatly alter the rate.

To near the saturation point, the oxygen supply to hemoglobin in general determines the rate of oxidation; *i.e.*, the time varies indirectly as the volume of oxygen-carrying liquid relative to hemoglobin.

#### THE STEADY STATE OF HEAVY WORK.

By L. J. HENDERSON, D. B. DILL, C. VAN CAULAERT, A. FÖLLING,  
AND THOMAS C. COOLIDGE.

*(From the Medical Laboratories of the Massachusetts General Hospital,  
Boston.)*

The behavior of the human mechanism working under a load which, though heavy, falls short of an overload, is not well known. Is there an analogy with the behavior of a machine which adjusts itself in a steady state no less smoothly under a heavy load than when idling?

The results of many experiments convince us that there is such an analogy. A single typical experiment on one of us may be taken as an example of the physiological adjustments which ordinarily accompany work. The subject rode a stationary bicycle for 68 minutes. During this period his mean oxygen consumption was about 2000 cc. per minute, a ninefold increase over his basal metabolism.

After 10 minutes the oxygen consumption became steady, ranging from 1960 to 2010 cc. per minute from that time to the end of work. During the same period, the ventilation varied from 39 to

41 liters per minute, the respiratory rate from 21 to 22 per minute, and the respiratory quotient from 0.91 to 0.96. The alveolar  $p\text{CO}_2$  ranged from 39 to 42.5 mm., the  $p\text{CO}_2$  of the oxygenated mixed venous blood from 63 to 66 mm., and the pulse from 140 to 144. These data indicate that the blood flow varied from 19 to 21 liters per minute while the output per beat varied from 134 to 148 cc., and that the blood flow and output per beat certainly did not vary in relative magnitude more than this.

Samples of venous blood were drawn 15 minutes and 60 minutes after work began. The height of the  $\text{CO}_2$  dissociation curve at 40 mm.  $p\text{CO}_2$  changed from the resting level of 48 volumes per cent to 45 after 15 minutes and to 47.2 after 60 minutes. The lactic acid concentration in these samples of whole blood was 15, 55, and 43 mg. per 100 cc. The estimated values of pH, of the arterial blood were 7.44, 7.38, and 7.42, respectively.

No oxygen debt was being contracted in the latter part of the experiment, for the  $\text{CO}_2$  curves were nearly normal and the lactic acid concentration was maximal early in the experiment. The R.Q. of excess metabolism during the last 20 minutes was about 0.98. It follows that, despite the production of over 500 calories within an hour, the fuel continued to be carbohydrate almost exclusively. The subject felt but little fatigue after stopping work and immediately proceeded with the equilibration of the blood.

Evidently, it is possible for the human machine to carry on smoothly while arterial blood and, therefore, cell environment remain approximately constant during heavy work.

#### **INFLUENCE OF FATIGUE ON THE HEAT PRODUCTION DURING MUSCULAR WORK IN OBESE, NORMAL, AND THIN SUBJECTS.**

BY CHI CHE WANG, SOLOMON STROUSE, AND EDITH A. SMITH.

*(From the Nelson Morris Institute for Medical Research of the Michael Reese Hospital, Chicago.)*

160 metabolic tests were made on twelve overweight, four normal, and eight underweight adult women between the ages of 16 and 26. 50 per cent of the subjects were nurses in training and the rest consisted of students, home girls, and laboratory workers. The average percentage deviation from normal weight for the three groups was +63.3 for the overweight, +1.4 for the normal, and -18.3 for the underweight subjects.

Tissot gasometer in connection with Haldane gas analysis apparatus was employed for the study of gaseous exchange. The work done was measured by a bicycle ergometer designed by Dr. C. Drinker of Harvard University. Fatigue was produced by prolonged riding on the ergometer with a resistance of 6 pounds at an average speed of 120 R.P.M. The speed and rhythm were controlled by a metronome. The energy spent on muscular work was calculated from the difference of heat production during exercise on ergometer with resistance and that without resistance. As control a basal test was taken in every case. The average basal metabolic rate for the obese group was 4.1 per cent below standard. The corresponding figures for the normal and underweight groups were 0.3 and 2.4 per cent below standard. The respiratory quotients were 0.764, 0.798, and 0.779 respectively.

The average percentage increase of heat production due to muscular work uncomplicated with fatigue was 367.8 for the obese, 226.3 for the normal, and 323.6 for the underweight group. The corresponding figures for the three classes of subjects complicated with fatigue were 382.4, 241.1, and 329.5. A slight increase in the heat production due to fatigue was thus indicated in all three groups. The average values for the mechanical efficiency of the three groups before fatigued were 28.7 per cent for the obese, 53.0 for the normal, and 44.4 for the underweight subjects and after fatigued the values were 26.1, 47.4, and 40.3, respectively. Thus the mechanical efficiency is the lowest in the obese and the highest in the underweight subjects and a decrease in mechanical efficiency due to the influence of fatigue is shown in all classes of subjects. In spite of the fact that the duration of exercise and the speed of revolutions were controlled, fatigue brought a slight decrease in both the amount and the duration of exercise in obese and in underweight groups.

Another point of interest in connection with this investigation is the difference in endurance in the three classes of subjects. With a resistance of 6 pounds and an average speed of 120 R.P.M. the average value in minutes for the duration of exercise by the obese group before fatigued was 6.7 as against 9.3 for the normal and 13.6 for the underweight groups.

A rise of respiratory quotient above the basal was found in every case during exercise. The degree of rise, however, varied

greatly in all groups, with a maximum of 45 and a minimum of 0.7 per cent. The increase of respiratory quotient was greater after fatigue than before fatigue in 79 per cent of the overweight subjects. In all the normal subjects and in 55 per cent of the underweight subjects a greater increase occurred before fatigue. The average respiratory quotients of the three periods of the overweight group are: basal 0.764, before fatigue 0.860, and after fatigue 0.884. The corresponding figures for the normal and the underweight groups are 0.798, 0.855, and 0.859, and 0.779, 0.899, and 0.876, respectively.

#### THE CONDITIONS UNDER WHICH CARBOHYDRATE IS OXIDIZED IN PHLORHIZINIZED DOGS.

By H. E. C. WILSON.\*

*(From the Department of Physiology, Cornell University Medical College,  
New York City.)*

Dogs were fed with a diet sufficiently high in carbohydrate so that the normal basal respiratory quotients, determined 18 to 24 hours after the last food, ranged from 1.00 to 0.90. When phlorhizin was administered subcutaneously to such animals, both in oil and dissolved in  $\text{Na}_2\text{CO}_3$  (to insure immediate absorption), 17 hours after food was last given, no differences were noted in the height of the respiratory quotients in the subsequent 16 hours from those obtained in the normal animal under similar dietary conditions. The effect of the drug on the kidney occurred a few minutes after its injection. The rate of fall in the respiratory quotient depends on the supply of carbohydrate originally available in the animal, but quotients well above the diabetic level are obtained 30 hours after the first injection of phlorhizin. On the 3rd day respiratory quotients approximating the diabetic value of 0.687 are always obtained.

In order to determine whether animals in which the diabetic respiratory quotients had been established had lost the power to oxidize glucose when given orally in small amounts, metabolism experiments were made following the administration of 16, 10, and 5 gm. of glucose. When 16 gm. were administered there

\* Research Fellow in Medicine of the Rockefeller Foundation.

was always an unmistakable rise in the respiratory quotients during the 2 hours thereafter, with a gradual decline to the diabetic level in 4 hours. The amount oxidized was between 1 and 2 gm. of glucose. In four experiments in which 10 gm. were given the increase in the height of the R.Q. was slight and did not reach the diabetic value in 4 hours. With the administration of 5 gm. of glucose no change whatever in the R.Q. was observed. These results lead to the conclusion that the non-oxidation of glucose in the phlorhizinized animals during fasting or after the administration of small amounts of carbohydrate is to be traced to the hypoglycemia induced by phlorhizin action on the kidney rather than to any intrinsic impairment of the carbohydrate-oxidizing capacity of the cells.

The quantity of glucose present in the animal at the beginning of the experiment can be calculated (1) from the sugar excreted over and above a D:N ratio of 3.65, and (2) from the sugar oxidized, as shown by the R.Q. in the hours following the administration of phlorhizin until the diabetic figure is reached. In one animal (Dog 27, weighing 10 kilos) the amount oxidized was 28.99 gm. and that excreted 21.93 gm., giving a total of 50.93 gm. (or 0.51 per cent of body weight) of glucose present as glycogen at the beginning. In Dog 41, weighing 13 kilos, the amount oxidized was 52.65 gm., that excreted 22.91 gm., and 3.29 gm. transformed into fat (the R.Q. during the first few hours of the experiment being over unity), giving a total of 78.87 gm. (or 0.61 per cent of body weight) of glucose originally present as glycogen.

#### THE ACTION OF PHLORHIZIN IN NEPHRECTOMIZED DOGS.

By HARRY J. DEUEL, JR., ADOLPH T. MILHORAT, AND  
J. E. SWEET.

*(From the Departments of Physiology and Experimental Surgery, Cornell University Medical College, New York City.)*

The problem was to determine whether administration of phlorhizin to fasting dogs causes a definite impairment of the ability of the cells to oxidize carbohydrate or whether the non-oxidation of glucose in phlorhizin glycosuria could be traced to a secondary condition, such as hypoglycemia arising from the effect of phlorhizin on the kidney. If the effect were purely renal, then the

phlorhizinized nephrectomized dog should show a normal reaction toward carbohydrate.

The dogs lived between 4 to 6 days after nephrectomy. Daily administration of phlorhizin subcutaneously, given simultaneously both in olive oil and dissolved in sodium bicarbonate, did not produce the characteristic hypoglycemia found in the normal phlorhizinized dog.

The respiratory quotients of the fasting phlorhizinized nephrectomized dogs were determined daily for 4 or 5 days. In no case was the diabetic quotient of 0.687 obtained. The values were usually 0.73 or higher and in no case below 0.72. The administration of 16 gm. of glucose to one of these dogs raised the R.Q. to 0.86 and 0.80 during 2 successive hours, results such as might have been obtained in fasting animals but not in dogs fasting and phlorhizinized.

If phlorhizin prevents the oxidation of glucose, one would expect that its administration to nephrectomized animals would be followed by a progressive rise in the blood sugar and a parallel rate of increase in the non-protein nitrogen. However, no increase above the slight hyperglycemic level always found after nephrectomy occurred until just before death, the latter result being also duplicated in the control animal receiving no phlorhizin. One can only conclude under these circumstances that the carbohydrate arising from protein in the phlorhizinized nephrectomized dog is oxidized.

If there were no oxidation of carbohydrate in the kidneyless animals receiving phlorhizin, one would find that the non-protein nitrogen of the blood—which is the best index of protein metabolism in the absence of the kidney—would increase at a progressively faster rate after the 1st day of phlorhizin than in a control animal receiving no phlorhizin but with the kidneys extirpated. In both instances, however, the rate of increase in the non-protein nitrogen was exactly the same for 4 days after phlorhizin and only increased at a slightly faster rate just previous to death. In no case did the injection of phlorhizin in these animals give rise to sufficient acetone so that its presence could be detected in the breath even 5 days after its first administration. We can invariably detect it with normal animals 3 days after phlorhizin has first been given.

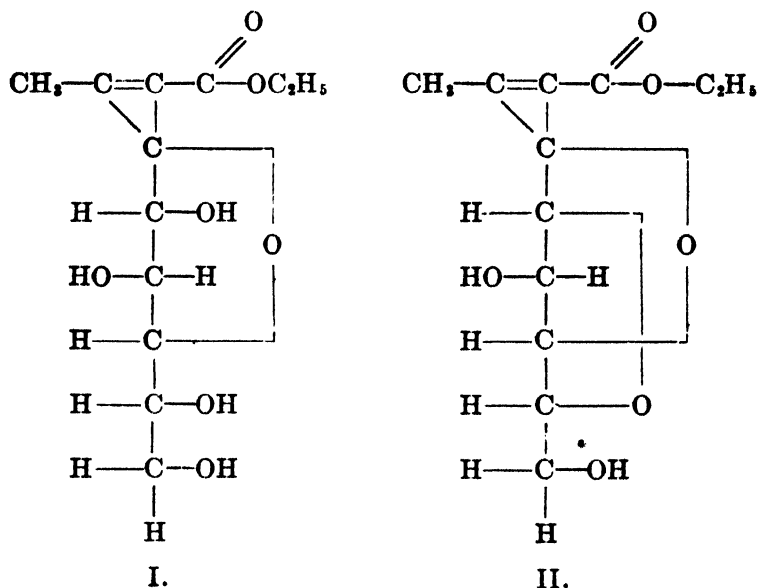
These results lead us to the belief that the non-oxidation of glucose during fasting and after small amounts of sugar in phlorhizinized animals with normal kidneys is not due to the impairment of the carbohydrate-oxidizing mechanism but simply to the secondary effects which all can be traced to its primary action on the kidney.

**FURTHER STUDIES IN ANTIKETOGENESIS. A NEW REACTION OF GLUCOSE. CONDENSATION WITH ACETOACETIC ESTER.**

BY EDWARD S. WEST.

*(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)*

Several condensation products of glucose and acetoacetic ester and acid have been obtained. Their chemical properties suggest the following tentative formulas for the mother substances:



A compound, III, isomeric with II, has been obtained. It has a free C=O group and powerful reducing action. Acids corresponding to I and II, and acetylated derivatives of I, II, and III have been prepared. The tetramethyl derivative of the acid from I is non-reducing. When, however, it is boiled in aqueous solution the carbon ring opens, the carbonyl group appears, and the compound reduces Benedict's alkaline copper solution in the

cold in a few minutes and more alkaline copper solutions practically instantly in the cold. In view of previous work of the writer this reducing action is probably due to the electron instability of the acetoacetic acid carbon atom as a result of linkage to the glucose molecule. Similar compounds may be involved in the mechanism of antiketogenesis in the animal body. Further work is in progress.

### A COMPARISON OF GLYCOLYSIS IN MUSCLE AND BLOOD IN VITRO.

By ETHEL RONZONI.

(From the Department of Internal Medicine, Washington University School of Medicine, St. Louis.)

A comparison of *in vitro* glycolysis of muscle and blood shows striking differences in the apparent mechanism of the reaction. In blood a determination of the balance between carbohydrate and lactic acid changes shows a loss of about 20 mg. of glucose which cannot be recovered as lactic acid. This amount is fairly constant and bears no relation to the amount of glucose involved in the reaction. That this failure of recovery of lactic acid is not due to loss by oxidation is shown by the fact that it occurs under anaerobic conditions produced by an atmosphere of nitrogen or by cyanide. Since the curve of CO<sub>2</sub> liberated by formation of acid checks with the lactic acid, this unaccounted for loss of carbohydrate is not accompanied by the formation of an acid end-product. There is no indication that this carbohydrate has become involved in a hexose phosphate combination since the inorganic phosphate increases during glycolysis. Fluoride which inhibits both glycolysis and the action of phosphatase would be expected to emphasize the esterification of hexose phosphates. It has no influence on phosphate changes in blood, which emphasizes the probability that phosphate changes in incubated blood are independent of carbohydrate changes.

In muscle the mechanism may be different. Davenport and Cotonio<sup>12</sup> have shown in muscle extract prepared according to Meyerhof<sup>13</sup> that more carbohydrate is lost than appears as lac-

<sup>12</sup> Davenport, H. A., and Cotonio, M., personal communication.

<sup>13</sup> Meyerhof, O., *Biochem. Z.*, 1926, clxxviii, 395.



tic acid. This extra loss of carbohydrate which is extensive in a phosphate buffer may be accounted for by a decrease in inorganic phosphate assuming the formation of a mixture of hexose mono- and diphosphate. We must also assume that hexose phosphate is not susceptible to the acid hydrolysis to which the tissues were subjected in the determination of total carbohydrate (Fürth and Marian<sup>14</sup>). We have confirmed the observation of Davenport and Cotonio.

In hashed muscle, however, the increase in lactic acid is considerably greater than the carbohydrate lost and cannot be accounted for by the hexose phosphate content of the muscle. If the increase in urea and ammonia nitrogen is a measure of the nitrogen metabolism, the carbohydrate from the protein metabolized will not account for the remainder of the lactic acid. At present the source of about one-quarter of the lactic acid produced by muscle *in vitro* is unaccounted for.

The curves of the rates of formation of lactic acid show different types of reaction in these two tissues. In blood the rate of lactic acid production is constant provided there is a concentration of at least 40 mg. per cent glucose. The accumulation of lactic acid has no effect on its rate of formation up to a concentration of 284 mg. per cent—the highest value we have obtained. This is less than half the amount produced in muscle. However, it is impossible to make this curve fit any portion of the typical logarithmic curve of muscle on muscle extract. The rate of lactic acid formation in muscle is rapid during the first 30 minutes, then slows down until the reaction is complete in about 2 hours. This is not due to a depletion of the carbohydrate but presumably to the accumulations of the end-products since if these are removed the reaction continues. In the case of muscle *extract*, it is possible since the concentration of lactic acid accumulated is small in comparison with muscle that the shape of the curve is due to the deterioration of the extract.

The effect of antiglyoxalase, prepared from dried pancreas as described by Dakin<sup>15</sup> which had previously been shown to inhibit the action of glyoxalase from rabbit liver on methylglyoxal, had

<sup>14</sup> Fürth, O., and Marian, J., *Biochem. Z.*, 1926, clxvii, 123.

<sup>15</sup> Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xiv, 423; 1913, xv, 177.

no inhibitory effect on lactic acid production in either muscle or blood. Presumably if the normal path of lactic acid formation was through methylglyoxal, it would be inhibited by this substance.

The fact that a 70 per cent alcoholic extract from desiccated pancreas described by Foster and Woodrow<sup>16</sup> inhibits lactic acid formation in muscle but not in blood suggests two distinct mechanisms for its production.

### OBSERVATIONS ON GLYOXALS AND GLYOXALASE, WITH A NEW COLORIMETRIC METHOD FOR GLYOXALS.

By N. ARIYAMA.

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)

The action of alkali cyanide on glyoxals gives very highly reducing substances which rapidly reduce many dyes and other oxidizing agents including the complex phosphotungstic acids. This reaction is made the basis of a colorimetric method for the determination of glyoxals.

On the addition of suitable amounts of arsenophosphotungstic acid (Benedict), sodium cyanide, and sodium carbonate, glyoxals give the stable blue color which indicates the presence of even so small an amount of glyoxals as 0.05 mg. with an accuracy of  $\pm 5$  per cent.

With this method the author has studied the rate of the conversion of glyoxal and methylglyoxal in buffer solutions of different pH. The glyoxals are quite stable at the neutral point but with increasing pH disappear more and more rapidly. At low pH (8 to 10) much of methylglyoxal which disappears is not converted to lactic acid. At higher alkalinity (0.1 N NaOH) the conversion to lactic acid is quantitative. The change of the glyoxals, however, does not directly mean the formation of corresponding hydroxy acids, at least at lower pH. The conversion of methylglyoxal to lactic acid is quantitative only in an excess of alkali.

The conversion of glyoxals by extracts of various tissues, *i.e.* the glyoxalase activity, also has been studied. The extracts convert methylglyoxal quantitatively to lactic acid. The opti-

<sup>16</sup> Foster, D. L., and Woodrow, C. E., *Biochem. J.*, 1924, xviii, 562.

mum pH for the enzyme activity lies in the neighborhood of pH 7. The glyoxalase activity of other animal tissues corresponds to 30 to 50 per cent of that of the liver.

Pancreas extracts inhibit the glyoxalase activity of other tissues (Dakin), but not completely. The inhibition is not due to insulin, which has no effect. The action of KCN upon glyoxals is catalytic. The product of this action upon methylglyoxal is not lactic acid, but its identity is unknown. A very high reduction potential develops when glyoxals are decomposed by KCN, but not when glyoxals are acted upon by glyoxalase or alkali.

#### ON COUPLED REACTIONS: THE INTERMEDIATE PEROXIDES IN THE OXIDATION OF FERROUS SALTS.

By PHILIP A. SHAFFER.

*(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)*

As relatively simple examples of "induced oxidations"—a class of reactions of great importance in biochemistry—the oxidation of ferrous salts by various oxidants has been studied with a view to evaluation of the relative oxidation intensities of the primary peroxide-like intermediates, which are presumably responsible for the coupled oxidation of acceptors. Data from (1) qualitative tests with different acceptors, (2) quantitative determination of ratios between acceptor and inductor oxidized, and (3) measurements of oxidation-reduction potential, indicate that the intermediates almost always have higher oxidizing intensity levels than the oxidant producing them; and that the intensity levels—and therefore the intermediates—vary with different oxidants. The phenomena of induced oxidation in these cases appear to be best accounted for by the hypothesis that the active intermediate is an unstable and transient molecular or ionic combination of reductant and oxidant, the complex thus partaking of the properties of both. In accord with this principle the oxidation of any substance by oxygen should give as a first stage a peroxide by the addition of one or more molecules of  $O_2$  and peroxide formation is known to accompany many oxidations of this sort. A new example is given in the following abstract. When this primary product breaks up a redistribution of valence electrons pre-

sumably occurs in response to the relative affinities of the several constituent atoms or molecules; with the result for example that  $\text{FeCrO}_4$ , the hypothetical first product in the reaction between  $\text{Fe}^{2+}$  and  $\text{CrO}_4^-$  ions, gives rise to  $\text{Cr}^{3+}$ ,  $4 \text{O}^-$ , and  $\text{Fe}^{5+}$ . Either the  $\text{FeCrO}_4$  or the  $\text{Fe}^{5+}$  ion may be regarded as the peroxide-like intermediate, which has higher oxidation intensity than  $\text{CrO}_4^-$  and thus is able to accomplish oxidations of substances not attacked under the same conditions by  $\text{H}_2\text{CrO}_4$  alone.

### OXIDATIONS INDUCED BY SUGARS.

By BEN K. HARNED.

*(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)*

Although the oxidation of sugars as "induced" by the oxidation of other substances such as ferropyrrophosphate and cerous salts has been studied, the fact appears never to have been noted that the oxidation of sugar by air may itself induce the oxidation of other substances. This latter phenomenon merits much biological interest in view of the prominence of sugar oxidation in living cells.

We find that the oxidation of sugars by air or oxygen (glucose and fructose in 0.3 N potassium hydroxide, fructose, and dihydroxyacetone in phosphate buffers) accomplishes the oxidation of other substances not affected by air alone. Arsenite and indigo carmine for example are thus oxidized by air at a rate paralleling the rate of sugar oxidation. Other acceptors will doubtless show similar behavior.

The reaction depends upon the activation of oxygen by the glucose. If the experiment is performed anaerobically the reducing power of the sugar disappears but no arsenite is oxidized, a fact showing that oxygen is necessary for the reaction. The reaction is also dependent upon the activation of the sugar. Fructose in 0.3 N potassium hydroxide induces the oxidation of arsenite very rapidly, less rapidly in molar phosphate-hydroxide, pH 11.1, and slowly in molar phosphate, pH 7.6. Only as the sugar is activated and becomes oxidizable does the induced oxidation occur. There are therefore two factors, the activation of the sugar and the presence of oxygen.

The effect of cyanide varies with the concentration, both acceleration and slight inhibition being observed. Some participation of traces of iron in the induced oxidation is not excluded, but the primary formation of a peroxide-like combination of sugar plus oxygen, appears to be the main factor in the reaction. The intermediate formation of such a peroxide is indicated not only by the apparent activation of one-half the oxygen absorbed, but by the fact that in the presence of barium hydroxide much barium peroxide precipitates. This is presumably formed by secondary reaction between the primary sugar-peroxide and barium hydroxide. The fact that the sugars exhibit this coupled oxidation even at moderate alkalinity (pH 7.6) suggests the probability that such occurs also in sugar oxidation in living cells.

When the solution contains 20 millimols of glucose per liter and 20 millimols of  $\text{As}_2\text{O}_3$ , 2 millimols of glucose accomplish the oxidation of 1 millimol of  $\text{As}_2\text{O}_3$  to  $\text{As}_2\text{O}_5$ , which demonstrates that under the conditions (aeration with oxygen or air in 0.3 N potassium hydroxide) 1 atom of oxygen is activated by 1 atom of glucose.

#### ELECTROMETRIC REDUCTION POTENTIALS OF SUGARS.

By P. W. PREISLER.

*(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)*

One aspect of the problem of biological oxidation appears to be the preliminary activation of metabolites under the influence of protoplasm with the production of highly reducing substances, the study of which began with Ehrlich on the reduction of dyes by tissues and is being continued by many investigators working upon biological or catalytic or enzymic dehydrogenation, and by Clark and others on reduction potentials. In view of the commonplace facts (1) that under the action of alkali sugars are transformed into strongly reducing substances some of which pass to lactic acid, and (2) that the oxidation of sugars is a prominent reaction in living cells, the question arises whether the activation of sugars may be one of the means by which cells exert their reducing power, or perhaps produce that negativity which appears to be an accompaniment of physiological activity. In the hope of obtaining data which might aid in judging this question we have attempted the quantitative evaluation of the reducing intensity of various sugars

in solutions of known pH. The method used is the potentiometric measurement of the reduction potentials registered by bright platinum and gold electrodes.

Although the transformations of sugars by alkali are, as a whole, irreversible reactions and the potentials therefore cannot be treated as equilibrium values conforming to the classical electrode equations, the values are, under certain conditions, fairly reproducible and significant. At high alkalinity the potentials rapidly attain values which are moderately constant over long periods and, within certain limits, are little influenced by concentration or the presence of such reversible systems as methylene blue, indigo sulfonate, or anthraquinone sulfonate.

At lower alkalinity the potentials are increasingly variable and uncertain unless poised systems such as the dyes above named are added. In the presence of these, the potentials rise to fairly definite maxima, which although in some cases doubtless registered by the dye are *caused* by the sugar and therefore characterize it, not the dye.

The maximum potentials attained correspond to an rH of about 6 to 8, at pH 10 to 13. With dihydroxyacetone and glycol aldehyde the rH decreases only slightly at lower alkalinity until pH 9, beyond which the results become much lower, uncertain, and variable. With glucose and other hexoses the rH decreases with decreasing pH, becoming uncertain below pH 10.

It is suggestive that the potentials measured for the sugars in strongly alkaline solution expressed in rH values (as defined by Clark) are of the same approximate order as the reduction potentials so far observed in hashed tissues. The data however warrant nothing more at present than to raise the question whether the two phenomena can be correlated.

#### POTENTIOMETRIC STUDIES OF THE OXIDATION AND REDUCTION OF EPINEPHRINE AND RELATED COMPOUNDS.

By EDWARD C. KENDALL AND EDGAR J. WITZEMANN.

(From the Section on Biochemistry, The Mayo Foundation, Rochester, Minnesota.)

Epinephrine and adrenalone have been shown to influence basal metabolic rate. The object of this investigation was to determine whether these compounds can act as cyclic catalysts. The

results may be summarized as follows: Ephedrine is not affected with any of the oxidizing agents used. Epinephrine its monomethyl, monoethyl and anhydride, adrenalone, amino, and dimethylaminoacetopyrocatechol are oxidized by dibromindophenol. Epinephrine and dimethylaminoacetopyrocatechol are not acted on with methylene blue. Adrenalone, amino, and dimethylaminoacetopyrocatechol are oxidized with indigo carmine. Adrenalone is reversibly oxidized and will act as a cyclic catalyst. The oxidized form can be reduced with mild reducing agents, such as reduced indigo. Amino and dimethylaminoacetopyrocatechol behave in a similar manner. Epinephrine, its monomethyl and monoethyl ether and anhydride derivatives, are not reversibly oxidized. Atmospheric oxygen will not oxidize adrenalone. Epinephrine will not act as a cyclic catalyst with atmospheric oxygen, resulting in the oxidation of adrenalone, except in the presence of a third component of the system.

Paradoxical results concerning the oxidation of these compounds indicate that the presence of chemical groupings is of greater significance than the intensity of the oxidizing compound. Dimethylaminoacetopyrocatechol is not oxidized at appreciable rate with methylene blue. It is oxidized with indigo carmine at a much faster rate. Adrenalone is not oxidized with atmospheric oxygen or hydrogen peroxide with or without traces of iron. It is readily oxidized with these agents if a small amount of indigo carmine is present. By constant potentiometric measurements the presence of a more intense oxidizing intermediate substance is excluded and the probable explanation is found in the properties of certain groupings which are formed as intermediate compounds. The speed of reaction is limited by the presence of these intermediate compounds and is not proportional to the intensity of the oxidizing compound.

#### THE FATE OF GLUCOSE IN ADRENALECTOMIZED RATS.

BY CARL F. CORI AND GERTY T. CORI.

*(From the State Institute for the Study of Malignant Disease, Buffalo.)*

The purpose of these experiments was to study the carbohydrate metabolism of animals without epinephrine secretion. Accessory medullary tissue has not been found in the rat. About 20 per cent of the operated rats 7 to 14 days after the complete re-

removal of both glands had either maintained their original body weight or even surpassed it. Only such animals were used for the experiments though the percentage of rats surviving double adrenalectomy was much greater. The animals were fasted for 24 hours. Nitrogen excretion and the amount of glucose absorbed, oxidized, and stored as glycogen in the liver and in the rest of the body was determined on the same animals. Control rats, fasted previously for 24 hours, were used for the determination of the preformed glycogen. In one series of experiments glucose alone was fed, in a second series 5 units of insulin were injected simultaneously with the sugar feeding. In both series of experiments glucose oxidation plus glycogen formation in the liver and in the rest of the body accounted for 86 per cent of the absorbed sugar. The insulinized animals (per 100 gm. of body weight per 4 hours) oxidized 98 mg. more glucose and deposited 87 mg. less liver glycogen than the animals without insulin. It will be noted that the amount of glucose that fails to be deposited in the liver of the insulinized animals corresponds to the amount of glucose that they oxidize in excess. Since the amount of glucose absorbed was nearly the same in both series of experiments, there was no marked difference in the amount of glycogen deposited in the rest of the body tissues. These results differ in no way from those obtained on rats with intact adrenals. The inhibition of glycogen synthesis in the liver of insulinized rats is, therefore, not due to the secretion of epinephrine. It is merely a secondary phenomenon due to the increased disposal of glucose in the muscles.

#### THE EFFECT OF THYROXIN ON A SUBJECT ON HIGH CARBOHYDRATE DIET.

BY IRENE SANDIFORD AND KATHLEEN SANDIFORD.

*(From the Section of Clinical Metabolism, Mayo Clinic and The Mayo Foundation, Rochester, Minnesota.)*

An 18 day metabolism experiment was presented in which was studied the effect of giving 7 mg. of thyroxin intravenously to a myxedematous subject who was on a diet containing 600 gm. of carbohydrate, 52 gm. of protein, and 110 gm. of fat. On this excessively high carbohydrate diet resulting in a basal respiratory quotient near unity and with a constant nitrogen intake of 8.3 gm. the total urinary nitrogen increased from an average of 8.0 gm.



to 13.9 gm. and remained markedly elevated for more than 10 days, averaging 12.2 gm. daily. The increase in total nitrogen was, as is usual after thyroxin, almost entirely due to increase in urea nitrogen. There was, however, a delay of 2 to 3 days before the increased urea elimination became manifest; also there was possibly a slightly greater delay than usual in the development of the increased heat production. The characteristic temporary increase in elimination of urea which accompanies the elevating of the thyroxin concentration of the tissues is, therefore, not abolished by an excessively high carbohydrate diet. These results are in harmony with the conclusion of Boothby, Sandiford, Sandiford, and Slosse that the temporarily increased urea elimination following the administration of thyroxin is a phenomenon of equilibration associated with the establishment of a new level of deposit protein conforming to the increased concentration of thyroxin in the tissues. This interpretation is consistent with the fact, shown by Boothby and Sandiford, that nitrogen equilibrium can be readily obtained, provided the diet is sufficient, in hyperthyroid individuals because in this condition the subject has had time to come into equilibrium with the new level of thyroxin concentration of the tissues. And this explanation is also in agreement with the fact established by Lauter and Jenke that it is possible to obtain a minimum nitrogen excretion in hyperthyroid patients on a high carbohydrate protein-free diet and with the finding of Deuel, Sandiford, Sandiford, and Boothby that the characteristic temporary effect of thyroxin on the nitrogen elimination is obtained after the nitrogen output is reduced to a minimum by a nitrogen-free diet containing 400 gm. of carbohydrate.

#### THE TRANSPORT NUMBERS OF SOLUTIONS OF FIBRIN IN DILUTE ACIDS AND ALKALIES.

By DAVID M. GREENBERG.

*(From the Division of Biochemistry and Pharmacology, University of California Medical School, Berkeley.)*

Transport numbers of fibrin in acid and alkaline solutions were determined according to the procedure developed for casein.<sup>17</sup>

<sup>17</sup> Greenberg, D. M., and Schmidt, C. L. A., *J. Gen. Physiol.*, 1924, vii, 287.

The fibrin used was prepared by dispersing washed and ground coagulated fibrin at 65°C. in solutions of about 0.02 N of either sodium hydroxide or hydrochloric acid. The solution was then neutralized and the precipitated fibrin was washed free from electrolytes. The purified fibrin had very different properties, depending upon whether it was dispersed in alkali or acid. The alkali-dispersed fibrin gave very viscous solutions and an electrochemical constant<sup>18</sup> of about 9.0; the acid-dispersed fibrin solutions were quite fluid and the electrochemical constant was found to be about 6.0. The transport numbers obtained for fibrin in sodium hydroxide, potassium hydroxide, and lithium hydroxide solutions are in accord with the results obtained for casein in these alkalies. They indicate independent migration of the charged fibrin and the respective cation. From the data an average value of 40 reciprocal ohms was calculated as being the mobility of the negatively charged fibrin at 25°C. The behavior of fibrin in solutions of the strong acids, hydrochloric, hydrobromic, and nitric, was found to be similar to its behavior in alkali solutions. The value obtained for the mobility of the positively charged fibrin, however, is surprisingly high, being about 77 reciprocal ohms, a value that is of the same magnitude as that of the potassium ion. Presumably the difference in mobility between the negatively and positively charged fibrin is to be explained by a difference in the degree of hydration. Transport number data of fibrin when dissolved in the weak acids, phosphoric, formic, and lactic, showed a different behavior than in solutions of the strong acids. The transport numbers of the fibrin are much higher than would be expected from its mobility in strong acids and the mobilities of the anions of the weak acids used. The explanation for this is that a certain number of the acid anions of these weak acids is held by the fibrin in a non-ionized form and carried along with the fibrin in the opposite direction to their normal course. This phenomenon is similar to that which was observed in solutions of casein with the alkaline earth hydroxides.<sup>19</sup> This difference in the electrochemical behavior of the two types of acids is probably

<sup>18</sup> For definition of this term see foot-note 17.

<sup>19</sup> Greenberg, D. M., and Schmidt, C. L. A., *J. Gen. Physiol.*, 1926, viii, 271.

of considerable significance in connection with other properties of proteins, such as swelling and osmotic pressure.

### STUDIES ON GLUTELINS.

By FRANK A. CSONKA AND D. BREESE JONES.

(*From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

It was reported previously before the Society<sup>20</sup> that wheat glutelins  $\alpha$  and  $\beta$  are precipitable with ammonium sulfate from an alkaline solution at a low ammonium sulfate concentration. By applying the proposed method to the preparation of glutelins of other cereals, such as rice, oats, and corn, we came to the conclusion that our method not only produces a more satisfactory separation of glutelins, but serves also for the characterization of glutelins as a class. Analyses of the two wheat glutelins and rice glutelin by the Van Slyke method showed pronounced differences regarding the amino acid content from those reported by others.

The oat prolamin is extractable quantitatively only by hot 70 per cent alcohol. Heating the flour with 70 per cent alcohol solution, however, largely denatures the glutelin and thus makes it slightly soluble in 0.2 per cent sodium hydroxide. It was found that by the addition of alcohol to the alkaline extract of the flour it was possible to precipitate the glutelin free from the prolamin by the addition of a very small quantity of ammonium sulfate. Purification of the glutelin by reprecipitation was necessary to make it entirely free from prolamins. Work is being continued on the physical and chemical behavior of glutelins in general.

### DETERMINATION OF ASPARTIC AND GLUTAMIC ACIDS IN PROTEINS.

By D. BREESE JONES AND OTTO MOELLER.

(*From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

The percentages of aspartic and glutamic acids in proteins as recorded in the literature are in nearly all cases too low, with the

<sup>20</sup> Jones, D. B., and Csonka, F. A., *Proc. Am. Soc. Biol. Chem.*, 1925, vii, 9; *J. Biol. Chem.*, 1926, lxxvii, p. ix.

exception of those for three or four proteins which have been recently analyzed by newly developed methods. Particularly is this true for aspartic acid. New determinations of these amino acids have been made in several typical proteins, and invariably higher results for aspartic acid were obtained than those previously obtained by the ester method. In several cases from 4 to 9 times as much was found. In the case of glutamic acid such wide differences were not found, especially in those cases where the old figures had been obtained chiefly by the separation of glutamic acid hydrochloride directly from the hydrolysates, and where the losses attendant upon the ester method of analysis were not involved.

The determination of these amino acids in various proteins was undertaken with two objects in view: (1) to obtain more accurate knowledge of the amounts present in the proteins; and (2) by adapting and modifying the more recent methods of analyses, to determine the conditions and sequence of analytical procedure that seem to give the most satisfactory results.

The results obtained are summarized in Table I.

TABLE I.

Protein.	Aspartic acid.	Glutamic acid.
	<i>per cent</i>	<i>per cent</i>
Glycinin (soy bean).....	9.39	.
Edestin (hemp seed).....	10.19	19.16
Ovalbumin (hen's eggs).....	6.22	13.27
Lactalbumin.*.....	9.30	12.89
Stizolobin* (velvet bean).....	9.23	14.59
Fish muscle (halibut).....	7.92	13.65
Ox muscle.....	5.88	13.45
Shrimp muscle.*.....	6.98	15.00
Gliadin (wheat).....	0.77	41.15
Glutenin ".....	2.03	23.48
Arachin (peanut).....	5.61	19.57
Kafirin* (kafir).....	2.27	21.23
Locust bark albumin.*.....	7.72	4.48

\* Although the figures for these proteins have appeared in previous publications from this laboratory, they are included in the table because they were determined largely by the newer methods.

**THE DETECTION AND ESTIMATION OF SULFHEMOGLOBIN.****BY WALTER R. CAMPBELL.**

*(From the Department of Medicine, University of Toronto, and the Toronto General Hospital, Toronto, Canada.)*

Among the more unusual types of cyanosis are those produced by alterations in the constitution of the blood pigment. Sulfhemoglobin and methemoglobin appear in the blood during infections with certain bacteria, in certain intestinal conditions, and in acute or chronic poisoning with coal tar drugs. Differentiation of these two pigments depends on spectroscopic examination of the blood. Both pigments have absorption bands in the orange-red of the spectrum and these can be differentiated by a calibrated spectroscope of good quality. Clarke and Hurtley's test—the shift in the absorption bands when acid-free carbon monoxide is added to sulfhemoglobin—is also specific, but difficult to make out by the smaller spectroscopes. Considerable reliance has therefore been placed on Van den Bergh's test—the gradual disappearance of the absorption band when ammonium sulfide is added to methemoglobin. If sulfhemoglobin is present the band is unaffected. We have found that the ammonium sulfide solution must be freshly prepared and must contain excess ammonia as older preparations containing polysulfides, of themselves, cause the production of sulfhemoglobin. A simpler method of differentiating methemoglobin from sulfhemoglobin is proposed dependent on the fact that when sodium cyanide solution is added to the blood, cyanhemoglobin is formed and the methemoglobin band, if present, is destroyed at once while the disappearance of a sulfhemoglobin band is very gradual (some minutes).

Since it has been found that sulfhemoglobin fails to yield any oxygen when potassium ferricyanide is added even though it is extracted in a vacuum, it may be estimated by the following technique. (1) The oxygen capacities of a sample of normal blood and of the cyanotic blood are determined either by the Van Slyke or the Haldane technique and the amount of hemoglobin free to combine with oxygen is calculated. (2) The standardized normal blood and the sulfhemoglobin blood are simultaneously converted to acid hematin solution and compared, after standing 15 minutes, in a colorimeter. The difference between the values for the ab-

normal blood obtained by the oxygen capacity method and the colorimetric method gives the amount of sulfhemoglobin present.

### THE REVERSIBILITY OF PROTEIN COAGULATION.

BY M. L. ANSON AND A. E. MIRSKY.

*(From the Biophysical Laboratory of the Cancer Commission of Harvard University, Boston.)*

It is generally supposed that the heat coagulation or denaturation of a protein is irreversible. Our experiments on a typical coagulable protein—hemoglobin—show that denaturation and coagulation are reversible reactions. The protein solution is denatured or coagulated by (1) heating in boiling water, (2) addition of excess HCl, (3) heating to boiling in presence of HCl, (4) allowing to stand in a concentrated urea solution. It is demonstrated in four different ways that the denaturation or coagulation has been complete.

From the denatured or coagulated product a protein solution is obtained which has been compared with the original native protein in the following ways: (1) It is heat-coagulable and the temperature of coagulation is the same as that of the native protein. (2) It is soluble in neutral solutions, in which the denatured or coagulated protein is practically insoluble. (3) Its color is the same as that of the native protein. (4) Its spectroscopic properties, when measured exactly, are the same. (5) It has the same derivatives. (6) It can be crystallized and the crystals have the same form and habit as has the native protein. (7) It not only has the above properties of a native protein, but is quantitatively (as shown *viz.* by gas affinity measurements) like the native protein of the same species from which it was prepared.

Since this protein is in all these respects indistinguishable from the native protein from which it was prepared, it is concluded that the denaturation or coagulation of hemoglobin has been reversed.

Since hemoglobin is a typical coagulable protein the coagulation of other proteins is probably reversible too.

A yield of about 40 per cent can be obtained.

We are investigating the biological implications of reversible coagulation and denaturation.

**THE CONDUCTIVITY METHOD AND PROTEOLYSIS.****I. EXPERIMENTS WITH PEPTONE.**

BY HARRY D. BAERNSTEIN.

*(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)*

The isoelectric point of Witte's peptone was determined ( $6.9 \pm$ ) by noting the effect of adding equal amounts of the material to a series of acetate buffer solutions.

If peptone be added to acid solutions of different hydrogen ion concentration, the conductivity increases if the pH is above 3, and decreases if the pH is below 3. The greatest change in conductivity by peptone is produced at pH 1.3. This is then the best acidity for following peptic digestion with the conductivity method.

In using the conductivity method in peptic digestions only the early stages of digestion are significant since relatively small quantities of peptone produced will increase the pH of the solution, and thus slow up the reaction.

**ON THE MECHANISM OF ENZYME ACTION.**

BY F. F. NORD.

*(From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul.)*

For a long time two view-points regarding the mechanism of enzymatic activity have profoundly influenced our conceptions in this field. To Oscar Loew is due the credit for the original suggestion that the enzymes possessing atomic groups with kinetic lability are able, even at a comparatively low temperature, to perform chemical action. This suggestion was later abandoned more and more, and we now generally assume that, as in the case of heterogeneous reactions, the reactants are adsorbed by the enzyme in order that reaction may ensue. In accordance with this we believe with Bayliss that the reaction velocity is determined by the concentration of adsorption complex, *i.e.* reactant-enzyme, present in the system. The possibility of carrying out such reactions depends doubtlessly on certain conditions of the surface,

which are also profoundly influenced by the hydrogen ion concentration.

Preliminary experiments carried out with certain zymase solutions which behave as lyophil colloids confirm this assumption. The reactivity of these solutions could be increased in such a manner that in laboratory tests the first stage of the reaction of zymase, for instance, on glucose produces 4, 5, and more cc. of carbon dioxide per minute, so that a cell-free zymase solution can easily be prepared, which will produce (using 20 cc. of the zymase preparation containing 5 per cent of the substrate) 100 cc. or more of carbon dioxide in less than 1 hour. The reactivity of the surface of the enzymes concerned in this reaction may be tremendously increased by appropriate peptization, and the velocity of the reaction later undergoes a decrease which cannot be due yet to the decreasing concentration of the reactant. Further experiments have shown that this capacity to react in an intensive manner can be maintained, within certain ranges, in both cases, by working with living cells or with the colloidal cell-free solutions. The experiments carried out indicate that certain chemical compounds are capable of forming an adsorption film<sup>21</sup> on the surface of the enzymes which has the rôle of a *protector*. It might therefore be assumed further that certain compounds which are supposed to have the effect of an activator of an enzymatic reaction are in reality not activating the reaction, but only insufficiently protecting the enzyme from the damaging effect of intermediate or final metabolic products of the reactions concerned. It might be regarded therefore as correct to assume that regardless of the absence or the presence of a protector, which might even be a specific protector, there is always a certain concentration of enzymes present which is potentially capable of acting. However, since the reactivity of the enzyme is dependent on its surface activity, it undergoes immediately with the initial reaction alterations which relatively decrease the velocity of the reaction independently of the concentration of the reactant. In the course of the reaction the ratio between active and damaged enzyme may decrease more and more below 1. Our present experiments have

<sup>21</sup> Nord, F. F., *Science*, 1927, lxxv, in press.



shown that it is possible to delay the speed of the reaction reflected in the change of the quotient noted above.

Since the most favorable conditions for the performance of an enzymatic reaction are in most cases not known, the statements above suggest the conclusion that in a great number of so called activations of enzymatic reactions by means of chemical compounds in fact no activation takes place by influencing enzymatic reaction through these compounds, *but the so called activators, which appear to be really protectors, enable the enzymes to act under conditions which are more nearly those which might be expected to be prevalent in ideal cases.*

The experimental work has been carried out in collaboration with Kurt W. Franke. The details will be prepared for publication in the near future.

#### SOME CHARACTERISTICS OF THE ENZYME ARGINASE.

BY ANDREW HUNTER AND JOSEPH A. MORRELL.

(From the Department of Biochemistry, University of Toronto, Toronto, Canada.)

The extremes of pH within which arginase possesses a measurable activity are rather sharply fixed at 4.7 and 11.5. This gives a total range of nearly seven units of pH.

As previously announced by ourselves<sup>22</sup> and independently discovered by Edlbacher and Bonem<sup>23</sup> the maximum of activity is situated at (or near) the unusually high pH of 9.8.

Since the activity increases through five pH units (4.7 to 9.8) and falls abruptly through two, the curve expressing its variations is strikingly unsymmetrical.

The longer ascending limb of this curve, instead of passing in one regular sweep from origin to summit, presents in the region between pH 7.5 and 8.5 a curious inflection, convex to the axis of pH. This makes it appear to be composed of two successive and nearly independent S-shaped curves of unequal size.

The whole curve describing the relation of arginase activity to pH is therefore quite unlike anything hitherto described in such a

<sup>22</sup> Hunter, A., and Morrell, J. A., *J. Soc. Chem. Ind.*, 1924, xliii, 691.

<sup>23</sup> Edlbacher, S., and Bonem, P., *Z. physiol. Chem.*, 1925, cxlv, 69.

connection. It has no resemblance to the dissociation curve of an acid or base, so that it does not seem possible to explain the observations by the simple assumption that the active part of the enzyme is a dissociation residue or a single dissociable ion. The fact that the rising limb of the curve could be interpreted as the titration curve of a polyvalent acid or base suggests a rather more complicated theory of the effect of reaction upon activity.

At a pH of 7.3 the temperature coefficient of arginase is 2.69 for the interval 0–10°, 2.32 for 10–20°, and 2.13 for 20–30°. The corresponding values of  $\mu$ —the constant of Arrhenius—are 15,300, 14,000 and 13,400. At 40° heat inactivation begins to be apparent; the coefficient for 30–40° drops as the reaction proceeds. At 50° the enzyme is at first slightly more, later distinctly less active than at 40°.

At no part of its course and under no conditions of temperature or reaction that have been studied does the hydrolysis of arginine by arginase obey the law of a monomolecular reaction. Under most conditions the first half or more of the reaction can be described by the equation

$$kt = m \log \frac{a}{a-x} - x$$

in which  $m$  is an empirical constant derived by computation from the experimental data.

### SOME PROPERTIES OF CRYSTALLIZED UREASE.

By JAMES B. SUMNER.

(From the Departments of Physiology and Biochemistry, Cornell University Medical College, Ithaca.)

The yield of urease crystals obtained by the author's method<sup>24</sup> can be increased somewhat by longer cooling, by cooling to a lower temperature, or by adding more acetone to the filtrate. The addition of more acetone is inadvisable. Certain samples of jack bean meal have been found to give poor yields of the crystals. It has been found that the crystals can be obtained by cooling 30 per cent alcoholic extracts of jack bean meal on ice for about 1

<sup>24</sup> Sumner, J. B., *J. Biol. Chem.*, 1926, lxi, 435.

week. When 2 per cent gum arabic as a diluent and protector is used, it has been possible to make accurate determinations of the activity of crystallized and of recrystallized urease. One lot of urease crystals showed an activity of 115,000 units per gm. of dry weight and after recrystallization an activity of 129,000 units. In the analysis the ammonia was estimated both by aeration followed by titration and by the method of direct Nesslerization. When direct Nesslerization is employed it is necessary to add as much of the urea-phosphate to the standard as is added to the unknown because urea-phosphate interferes with the development of color.

In addition to the list of substances that have been already shown to protect crystallized urease from inactivation by distilled water,<sup>25</sup> it has been found that protection is afforded by sodium acetate-acetic acid buffer at pH 6.1, by carbonic acid, and to some extent even by sodium sulfate solution. Solutions of trypsin also protect, while boiled trypsin has much less protective action.

#### THE AUTOLYTIC POWERS OF *BACILLUS COLI COMMUNIS*.

By E. GORDON YOUNG.

(From the Department of Biochemistry, Dalhousie University, Halifax, Canada.)

Highly concentrated emulsions of *Bacillus coli communis* were prepared in saline from growths on nutrient agar. They were then frozen and thawed repeatedly by means of a mixture of solid carbon dioxide and ether until the medium was rendered sterile or nearly so. The resulting medium was passed through a Berkefeld filter, then added to a solution containing glucose, peptone, and appropriate salt mixture. This medium was placed in a thermostat at 37°C. for several weeks in the presence of nitrogen gas and analyzed at frequent intervals. *Bacillus coli communis* has no autolytic power to decompose glucose. Autolysis of peptone proceeds very slowly at pH 6 to 8 as shown by an increase in the number of free amino groupings.

<sup>25</sup> Sumner, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 287.

**THE PREPARATION AND USE OF COLLOIDAL CARBON SOLUTIONS.**

BY JOSEPH M. LOONEY.

(From the Laboratory of Physiological Chemistry, Jefferson Medical College, Philadelphia.)

Colloidal carbon solutions were prepared by passing an electric current of between 3 to 4 amperes at a difference of potential of 20 to 30 volts between two soft carbon electrodes in a 0.03 N chromic acid solution. The resistance of the bath is used to regulate the current and it must be kept at about 80° by cooling.

The clear dark-colored solution obtained after allowing the heavy particles to settle is used. This solution will keep indefinitely.

The spinal fluid is used in dilutions of from  $\frac{1}{2}$  to  $\frac{1}{17}$ . 1 ml. of diluted spinal fluid and 5 ml. of the carbon solution are used in the test.

The normal spinal fluid causes a precipitation in the first two tubes only. The paretic fluid causes no precipitation in these two tubes and a curve resembling that given by Lange colloidal gold in the remaining tubes.

**CHEMICAL STUDY OF THYMUS INVOLUTION.**

BY FREDERICK S. HAMMETT.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

Thymus tissue from female albino rats 20, 30, 40, 50, 60, 70, 80, 90, 100, 115, 130, 150, 175, and 200 days of age was allowed to autolyze for 6 hours at  $37.5^{\circ} \pm 0.04$  in 0.7 per cent NaCl solution buffered to pH 7.4 with phosphate mixture. Initial and terminal amino acid nitrogen values were ascertained according to Van Slyke. Trichloroacetic acid was used as protein precipitant.

In this series of animals thymus involution, as evidenced by decreasing weight on age, began in the group 100 days old. An increase in the rate of *in vitro* autolysis appeared coincident with the appearance of involution. As the weight of the thymus decreased with age, the rate of *in vitro* autolysis increased.

This suggests, but is not proof for, the idea that the age involution of the thymus is in part, at least, endogenously conditioned.

**THE EFFECT OF THE PARATHYROID HORMONE ON GASTRIC SECRETION. THE CALCIUM CONTENT OF GASTRIC JUICE.**

BY W. C. AUSTIN.

*(From the Department of Physiological Chemistry, Loyola University Medical School, Chicago.)*

Overdosage with the parathyroid hormone has been shown to cause bleeding in the stomach in the fundic region. The effect of the parathyroid hormone on the gastric secretion of four female Pavlov pouch dogs was tested. Before administering the hormone preliminary control studies were made to ascertain the secretory responses of these animals to injections of 0.5 or 1.0 mg. of histamine dihydrochloride, dissolved in 1.0 cc. of normal saline. The volumes and acidities of the gastric juice samples were determined. The calcium content of the gastric juice and of the blood plasma was also estimated.

After data on fourteen control experiments was secured the animals were injected with parathormone. When the blood calcium was elevated the gastric responses of the animals to histamine were again determined, and the calcium content of the juice samples estimated.

In one animal the gastric response to histamine during hypercalcemia was 50 per cent below normal, while the other three animals showed no change in secretion with onset of hypercalcemia. The results with the first animal were probably due to the more prolonged dehydrating influence of the hormone and to a restriction of water intake. In all of the animals used the Pavlov pouch continued to secrete until the beginning of hemorrhage into the stomach, when it stopped completely and suddenly.

Analyses of eleven samples of gastric juice from normal dogs showed an average of 6.08 mg. of calcium in 100 cc. Analyses of twelve samples of gastric juice from hypercalcemic dogs showed an average of 6.65 mg. of calcium in 100 cc. The values were usually between 5.0 and 6.5 mg. of calcium in 100 cc. of juice.

**THE EFFECT OF SODIUM BENZOATE ON THE GROWTH OF RATS.**

BY WENDELL H. GRIFFITH.

*(From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis.)*

The rate of growth of young white rats on a diet containing sodium benzoate had been used as an index of the rate of formation of the amino acid, glycine, which is required for the detoxication of benzoic acid as well as for the synthesis of new tissue proteins. Normal growth occurred on a diet containing 2 per cent sodium benzoate and 12 per cent casein. On this diet 65 per cent or more of the combined benzoic acid present in the urine was benzoyl glycine, the remainder probably being benzoyl glycuronic acid. Rats failed to grow on diets containing 3 per cent sodium benzoate and 12, 35, or 50 per cent casein. Since the addition of 1 equivalent of glycine (1.56 gm.) to the diet containing 12 per cent casein and 3 per cent benzoate resulted in normal growth, it was evident that the previous failure of the rats to grow was due to a lack of glycine and not to the inability to form hippuric acid. Normal growth also occurred on diets containing 3 per cent benzoate and 60 per cent casein, or 35 per cent casein plus 0.78 per cent glycine, or 35 per cent casein plus 4.2 per cent gelatin. Rats failed to grow when alanine was substituted for glycine. In these experiments, 4.5 gm. of casein nitrogen did not successfully replace 0.3 gm. of glycine nitrogen and it was therefore concluded that casein was unable to supply precursors of glycine. This investigation of the formation of amino acids in the animal organism is being continued and extended.

**OBSERVATIONS UPON STARCH.**

BY C. L. ALSBERG AND E. P. GRIFFING.

*(From the Food Research Institute and the Department of Chemistry, Stanford University, California.)*

Mechanically grinding starch dry very greatly changes its properties. The greater part of such a starch becomes colloiddally soluble in cold water and does not yield a paste on heating. The viscosity of a starch paste is therefore dependent upon the intactness of the starch granules, and the viscosity of the paste is not a

true viscosity at all, but the plasticity of a suspension. Grinding a paste converts it into a syrup. Starch granules that have been injured by grinding swell in cold water and partly disperse spontaneously. Injury to the starch granule can be recognized by staining with an ordinary dye, such as Congo red. Intact granules do not stain. Ground starch dispersed in water is attacked almost as readily by hydrolyzing enzymes, such as diastase, as boiled starch. Most of the studies on the resistance of raw starch to diastase are without value, because practically all starch preparations contain greater or lesser numbers of injured granules which are readily attacked. Moreover, it can be shown by actual measurements that starch granules begin to swell at temperatures below 50°C. Swollen granules are therefore present when the action of diastase on raw starch has been studied at temperatures of about 40°C. and over. From ground starch clear solutions can be obtained which show but a slight Tyndall phenomenon. By a modification of the method of Beijerinck, well defined microscopic crystals may be obtained in the form of needles arranged in clusters in the shape of hemispheres. These are colored blue with iodine and show distinct birefringence when viewed with crossed nicols. Thus the birefringence of natural starch granules is probably due to an intrinsic property of its crystalline structure.

#### A STUDY OF THE LIPOIDS OF TUBERCLE BACILLI.

By R. J. ANDERSON.

*(From the Department of Chemistry, Yale University, New Haven.)*

The lipoid material, including neutral-fats, phosphatides, and wax, of tubercle bacilli was extracted with a mixture of alcohol and ether followed by chloroform treatment of a large quantity of fresh cultures of bacilli. The resulting extracts were examined separately.

The alcohol-ether-soluble constituents were fractionated by means of acetone into three groups consisting of fat or glycerides, phosphatides, and a small amount of wax. An appreciable quantity of a polysaccharide was also isolated from the alcohol-ether extract together with a small amount of basic substances. The chloroform extract consisted principally of wax.

The phosphatide fraction constituted a surprisingly large proportion of the lipid material, amounting to some 5 per cent of the dry bacilli. This fraction has been studied more closely and was found to represent a new type of phosphorized fat. On hydrolysis it yields some 67 per cent of fatty acids consisting of unsaturated and saturated acids, phosphoric acid, and about 33 per cent of a mixture of carbohydrates containing about 14 per cent of glucose.

The phosphorus-containing substance is apparently not a phosphatide since it contains no glycerol and only a trace of base in the form of ammonia which is probably bound on the phosphoric acid. It represents a new type of phosphorized fat which provisionally may be called a phosphosucride.

The amounts of the principal fractions that were obtained from tubercle bacilli are summarized below:

	gm.
Fat.....	240
Phosphosucride.....	194.5
Wax.....	485.6
Polysaccharide.....	33.9
Dry bacterial residue.....	2902.0

The chemical composition and the biological activities of these various fractions are now being investigated.

#### THE AVAILABILITY OF DISULFIDE ACIDS AS SUPPLEMENTING AGENTS IN DIETS DEFICIENT IN CYSTINE.

BY BEULAH D. WESTERMAN AND WILLIAM C. ROSE.

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.)

The inability of taurine to replace cystine in the diet<sup>26</sup> suggested the desirability of determining whether other sulfur compounds, particularly those containing the disulfide linkage, are capable of meeting the demands of the organism when the ration contains an inadequate amount of cystine. Accordingly, we have undertaken the synthesis of a series of compounds more or less closely related

<sup>26</sup> Beard, H. H., *Am. J. Physiol.*, 1925-26, lxxv, 658. Lewis, G. T., and Lewis, H. B., *J. Biol. Chem.*, 1926, lxi, 589. Rose, W. C., and Huddleston, B. T., *J. Biol. Chem.*, 1926, lxi, 599.



in chemical structure to cystine, and a study of the nutritive value of each substance by means of growth experiments in rats. To date, the physiological behavior of two synthetic products, namely dithiodiglycollic acid and  $\beta$ -dithiodipropionic acid, has been tested. The results of the experiments show conclusively that neither is capable of replacing cystine to the slightest degree. Despite the close similarity in chemical make-up of cystine and  $\beta$ -dithiodipropionic acid they behave entirely differently in the animal organism.

Our results serve to emphasize a fact brought out in a previous communication<sup>27</sup> from this laboratory; namely, that the animal organism is exceedingly exacting in its demands. The replacement of the amino groups of cystine by hydrogen atoms suffices to transform this essential component of the diet into a physiologically worthless substance.

The above studies are being continued with other compounds.

#### THE RELATION OF VITAMIN E TO IRON ASSIMILATION.

By NINA SIMMONDS, J. ERNESTINE BECKER, AND  
E. V. McCOLLUM.

*(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)*

Ferrous sulfate is shown to be harmful to rats when included in the diet in amounts as small as 0.2 per cent of the food mixture. It does not serve as a satisfactory source of iron for the rat, and when our Salt Mixture 20 is employed as a sole source of iron in the diet, the animals ultimately reach a condition which is followed by speedy decline in weight, marked enfeeblement, and in most animals the development of ophthalmia which we have designated as "salt ophthalmia."

There are two ways in which rats can be caused to recover from this crisis. One is by giving them wheat germ oil and the other is by replacing the ferrous sulfate of the diet by ferric citrate. Ferric salts are far superior as a source of iron to growing rats than are any ferrous salts yet examined.

A new interpretation of the function of vitamin E is offered. It is shown to be in some manner associated with iron assimilation.

<sup>27</sup> Cox, G. J., and Rose, W. C., *J. Biol. Chem.*, 1926, lxviii, 781.

It is suggested that the death of the fetuses in rats on diets deficient in this vitamin is due to a crisis in their iron assimilation, which can be obviated by providing the vitamin E in appropriate amounts from the beginning of pregnancy.

Liver fats contain vitamin E in considerable amounts, and liver contains much iron. It is suggested that the special value of liver in the diet recommended by Minot and Murphy lies in its content of vitamin E and of iron.

#### THE NATURE OF THE MATERIAL IN LIVER EFFECTIVE IN PERNICIOUS ANEMIA. I.

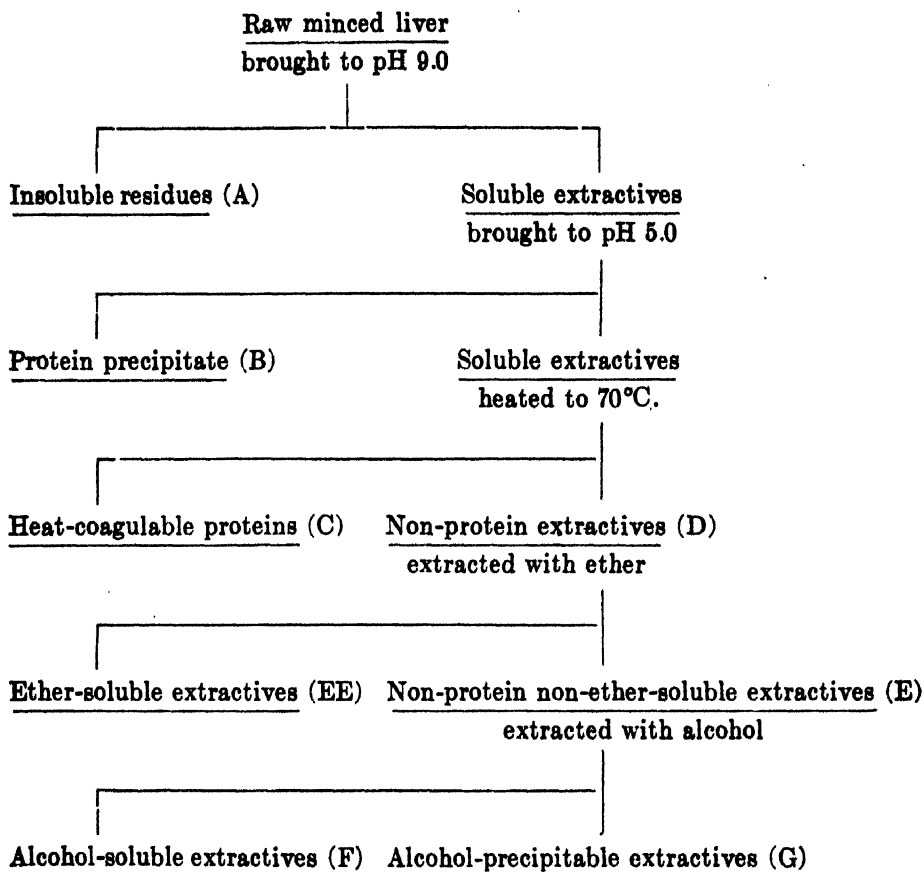
By EDWIN J. COHN, GEORGE R. MINOT, JOHN F. FULTON,  
HERMANN F. ULRICHS, FLORENCE C. SARGENT,  
JOHN H. WEARE, AND WILLIAM P. MURPHY.

*(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, the Medical Services of the Collis P. Huntington Memorial Hospital of Harvard University and the Peter Bent Brigham Hospital, Boston.)*

The feeding of liver has been shown to affect profoundly various bodily processes. It influences the growth of animals, the regeneration of hemoglobin, and the course of certain diseases. Recently Minot and Murphy have demonstrated that a diet containing large amounts of liver can markedly increase the number of red blood corpuscles in cases of pernicious anemia. During a relapse in this disease the ingestion of liver appears to cause the rapid maturation of the megaloblasts crowding the bone marrow. The response to the feeding of liver is first reflected in the peripheral blood by a temporary increase in the number of reticulated red blood corpuscles, followed by a marked rise in the total number of red blood corpuscles and in the concentration of hemoglobin.

The nature of the constituent, or constituents, of liver effective in pernicious anemia is being investigated by employing the aforementioned criteria and substituting for the whole liver a fraction thereof. The liver may be divided not only into its water-soluble and insoluble components, but also into its protein and non-protein, its lipoid and non-lipoid, its dialyzable and non-dialyzable constituents. The chemical dissection of the liver that we have

attempted is diagrammatically represented in the accompanying scheme.



The minced tissue of beef liver was first rendered alkaline to pH 9—at which reaction Bradley has shown that autolysis is inhibited—and the soluble extractives filtered. The insoluble residues are termed (A) in the accompanying chart. They contain, among other things, connective tissue and the water-insoluble proteins and fats.

The water-soluble extractives were then brought to pH 5 and the copious characteristic liver protein (B) precipitated in the neighborhood of its isoelectric point. The protein precipitate (B), and the water-insoluble residues (A), were rendered relatively free of each other, and of liver constituents soluble at both pH 5 and 9 by repeated extraction and precipitation. The characteris-

tic liver protein resembles casein in its general behavior, and may be purified by methods analogous to those employed in its isolation. The protein fraction (B) has been fed in large amounts to one patient with pernicious anemia for 13 days, and together with fraction (A) to another patient for 10 days without the occurrence of any particular change either in the number of reticulocytes or red blood corpuscles.

The solution from which the liver protein precipitated contained proteins. These represent in large part the albumins and globulins of the blood. They have been largely removed by heat coagulation at 70°C. These proteins (C) have not yet been incorporated in a diet.

The filtrate from (C) representing the non-protein extractives (D) has been concentrated *in vacuo* at 60°C. This concentrated liver extract (D) has been prepared as a powder, a viscous syrup, containing but 25 per cent of water, and as a solution. It has been added to the diet of four pernicious anemia patients.<sup>28</sup> A prompt increase of reticulocytes occurred, followed by a marked increase of the red blood corpuscles in the three who continued with this fraction. This and the subsequent effective fractions are essentially free of blood sugar-reducing substances.

But 2 per cent of the solids in (D) are soluble in ether, and their removal did not destroy the effectiveness of the residuum (E) which was fed to one patient.

Approximately 30 per cent of fraction (D) is soluble in strong alcohol. In practice a concentrated aqueous solution of (D) has been poured into such an amount of absolute alcohol as to render the final concentration approximately 95 per cent. The alcohol-soluble extractives (F) contain substances capable of reducing blood pressure. The precipitate (G) has been fed in daily doses of 9 to 14 gm., to three patients who promptly and rapidly improved. This fraction (G) has been shown to contain the active material effective in pernicious anemia. It has been freed from such lipoids as are soluble in ether, acetone, and strong cold alcohol. The solubility of these extracts has been studied in more

<sup>28</sup> The clinical aspects of these cases will be considered in another communication. One of the patients was at the Massachusetts General Hospital.

than twenty organic solvents. Certain of the constituents insoluble in the above solvents are soluble in normal butyl alcohol, in pyridine, and in glacial acetic acid.

The concentration of the extracts thus far employed is partially revealed by the analyses given in Table I. Besides being freed from lecithin and ordinary lipoids, and all but a trace of protein, they have been freed from all but a trace of sulfur and iron.

TABLE I.  
*Analyses of Liver Fractions Effective in Pernicious Anemia.*

Liver fraction.	Volume of extract fed per day.	Amount fed per day.	Solids in extract.	Nitrogen* in preparation.	Phosphorus in preparation.	Ash in preparation.
	cc.	gm.	per cent	per cent solids	per cent solids	per cent solids
II D	200	21.6	6.3	4.2		30.0
III D	200	10.8	5.4	7.6	3.2	29.3
IV D	100	15.7	15.7	4.6	2.2	19.6
V D	100	16.5	16.5	5.3	3.1	21.6
VI D	100	11.9	11.9	5.5	3.6	28.1
VII D	100	19.4	19.4	7.2	2.4	19.1
VIII D	100	14.1	14.1	5.1	2.2	16.7
VII G	50	9.8	19.6	6.9	3.0	20.5
VIII G	80	10.1	11.4	6.9	3.5	20.3
IX G	80	11.0	6.9	8.8	3.2	16.0
X G	25	9.5	38.0	6.3	1.7	20.8

\* Non-protein non-ammonia nitrogen.

### A NEW DIETARY DEFICIENCY PRODUCED WITH HIGHLY PURIFIED DIETS.

BY HERBERT M. EVANS AND GEORGE O. BURR.

(From the Department of Anatomy, University of California, Berkeley.)

In the course of our work on vitamin E it became necessary some years ago to attempt to withdraw all traces of this substance from dietaries. A new dietary deficiency appears when highly purified ingredients are used. Commercial casein, corn-starch, and lard may all contain the impurities which allow diets composed of them to produce female rats equaling in size and ovulation performance their sisters reared on natural foods. On the other

hand, diets of high purity rigidly stop growth at some time during the period of main ascent of the normal growth curve and females reared on them are greatly delayed in sexual maturity. Yet such dwarfed animals maintain glossy coats and apparent physiologic well being. The replacement of the extracts withdrawn from the casein, the substitution of crude casein for pure casein, or the addition of small supplements of liver or lettuce promptly produce growth and ovulation. Doubling the already high dose of yeast and cod liver oil does not do this. The advantage of this diet for other nutritional studies is pointed out.

#### VARIATIONS IN THE ANTIRACHITIC EFFECT OF ULTRA-VIOLET IRRADIATION.

BY H. STEENBOCK, E. B. HART, BLANCHE RIISING, AND  
C. A. HOPPERT.

*(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)*

During the last 2 years we have investigated the possibility of the increase in antirachitic value of cow's milk by direct action of sunlight on the animal, and have come to the conclusion that the effect, if any, has no practical significance from the standpoint of good milk production. Even intense irradiation of cows with artificially produced ultra-violet light from a quartz mercury vapor lamp increased the antirachitic properties of the milk to such a limited extent that it was barely detectable in rat experiments continued over a 5 weeks period.

With goats, on the other hand, we have succeeded in duplicating our former results. The antirachitic value of goat milk was practically doubled. Whether this difference in the reaction of the cow as compared with the goat is correlatable with difference in body area, with respect to volume of milk produced, or with secretory function of the skin or translocation of activated compounds, we are unable to say.

We have confirmed the conclusion of Meigs and coworkers that nervous reactions may impede calcium assimilation. With continued confinement of the goat in the metabolism cage as well as continued irradiation, the animal lost more and more lime in its fecal excretion as time went on. This occurred when the animal

became more restless as indicated by continued bleating and excitability. Ultra-violet radiation of such intensity as to double the antirachitic value of the milk was not able to prevent this excessive loss of lime.

#### **THE INFLUENCE OF DIETS CONTAINING UNSATURATED ANIMAL FATS ON REPRODUCTION AND LACTATION IN THE RAT.**

By MARY M. CLAYTON.

*(From the Department of Vital Economics, the University of Rochester, Rochester, New York.)*

As a part of the study of the efficiency of various proteins (meat, milk, and egg) at different levels for reproduction and lactation in the rat, a series of rations containing lard for adjusting the fat content and cod liver oil for a supply of vitamins A and D was compared with parallel series without lard and with separate daily administration of cod liver oil or radiation.

From observations on several hundred animals, many of them in the third generation, it is clear that the presence of these and perhaps other unsaturated animal fats in the ration seriously interfered with reproduction. Reproduction but not lactation was improved when these fats were omitted or administered daily. The presence of wheat germ oil in the fat-containing rations favored reproduction and also lactation, especially in skim milk powder rations. Depending on as yet unknown factors various rations differ considerably in their ability to protect vitamin E from the destructive action of unsaturated animal fats.

#### **VITAMIN REQUIREMENTS OF THE NURSING MOTHER.**

##### **I. THE PRODUCTION OF BERIBERI IN THE NURSING YOUNG OF THE ALBINO RAT ON DIETS ENTIRELY SATISFACTORY FOR GROWTH.**

By BARNETT SURE AND S. J. SCHILLING.

*(From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville.)*

Lactation studies on synthetic diets containing alcoholic extracts of wheat embryo as the only source of vitamin B disclosed the fact that the requirements of such extracts for normal mam-

mary gland function are at least twice as great as that for optimum growth. In connection with further studies on the rôle of vitamin E in fertility and lactation we have had occasion to vary not only the amount of wheat oil, but also the proportion of defatted wheat embryo in the ration. On a ration consisting of casein 20, Salts 32 4.0, ferric citrate 0.25, acetone-extracted wheat embryo 30.0, wheat oil 3.0, dextrin 42.75, and 0.3 cc. of cod liver oil per animal per day, excellent results in fertility and lactation were secured. During a reproduction period of 204 days, three females delivered ten litters, and out of 52 young allowed to be reared, 50 were successfully weaned—a lactation efficiency index of 96 per cent. Reducing the concentration of defatted wheat embryo in the ration to 20.0 per cent (10 per cent dextrin replacing an equivalent amount of wheat embryo) resulted in considerable infant mortality. During a reproduction period of 200 days, three females gave birth to ten litters, and out of 59 young allowed to be reared, only eleven young were successfully weaned—a lactation efficiency index of only 18 per cent. Most of the young died during the latter part of lactation with typical symptoms of beriberi. While some of the young died during the night, and in such cases autopsies were not made until the following morning, we have had occasion to secure necropsy findings on a good many nursing young immediately after death. Out of thirty-five young examined the stomachs of thirty-two were found to be well distended with coagulated milk. The young succumbed when mothers were in active state of milk secretion. In such animals we observed with a high degree of regularity hemorrhagic appearances in the cranial and digital bones, caudal and thoracic vertebræ, ribs, sternum, femur, and tibia, as well as in the kidney, liver, and thymus. The hemorrhagic condition was most marked and most frequently noted along the junctures of the parietals with each other and with the occipital bone.

In the absence of vitamin E in the ration, even on the high levels of defatted wheat embryo, resorptions of first litters only were obtained.

As little as 10 per cent of acetone-extracted wheat embryo in the ration as a source of vitamin B served for excellent growth. The results of this investigation show that the vitamin B requirements for successful lactation are considerably greater than that



for optimum growth; also that the derangement in milk secretion, when the diet of the nursing mother contains inadequate proportions of vitamin B, is to be attributed to inferior quality rather than insufficient quantity of milk.

### THE TRANSMISSION OF ULTRA-VIOLET LIGHT BY A GLASS SUBSTITUTE.

BY WALTER C. RUSSELL AND O. M. MASSENGALE.

(From the Department of Agricultural Biochemistry, New Jersey State Agricultural Experiment Station, Rutgers University, New Brunswick.)

According to a chemical measurement by means of the acetone-methylene blue reaction,<sup>29</sup> a glass substitute<sup>30</sup> was found to transmit 47 per cent of the light, of wave-length less than 3200 Å., from a quartz mercury lamp. This value was used as a guide in a biological test in which the percentage of ultra-violet light transmitted, which is effective in normal bone formation in the chicken, was determined.

Pens of chickens were fed a leg weakness-producing ration (99 per cent yellow corn, 1 per cent sodium chloride, and skimmed milk *ad libitum*)<sup>31</sup> and given a daily dosage of ultra-violet light from a quartz mercury lamp at a distance of 3 feet. One set of pens received unfiltered light and was compared with the pens which received the radiation through the glass substitute. The ash, and in some cases the calcium and phosphorus, of the dry, alcohol-ether-extracted femurs and wing bones, were determined in four to eight birds selected from each pen, each week, for an 8 or 10 week period. These values were used as a measure of the effectiveness of the rays in bone formation.

1, 10, 15, and 20 minute periods of irradiation daily with direct light were compared with 2½, 25, 45, and 60 minute periods in which the light was passed through the glass substitute. 1 minute of direct and 2½ minutes of filtered light were found to be inadequate. 25 minutes of filtered light were found to be a little less effective than 10 minutes of direct and neither one was quite

<sup>29</sup> Webster, A., Hill, L., and Eidinow, A., *Lancet*, 1924, i, 745.

<sup>30</sup> Cel-O-Glass supplied by Acetol Products, Inc., New York.

<sup>31</sup> *Wisconsin Agric. Exp. Station, Research Bull.* 371, 1925, 21. Hart, E. B., Steenbock, H., and Lepkovsky, S., *J. Biol. Chem.*, 1925, lxx, 571.

adequate. 45 minutes and 60 minutes of filtered light were found to be ample and equivalent to 15 and 20 minutes of direct light respectively. Therefore, the percentage of the effective rays which pass through this glass substitute is greater than 33 per cent but less than 40 per cent. Control pens were used with cod liver oil and with the basal ration, only. The data have been obtained on about 900 birds.

#### **VITAMIN D IN EVAPORATED MILKS MADE BY VACUUM AND AERATION METHODS.**

By HANNAH E. HONEYWELL, R. ADAMS DUTCHER, AND  
C. D. DAHLE.

*(From the Department of Agricultural and Biological Chemistry, Pennsylvania State College, State College.)*

Five types of milk have been studied for their antirachitic potency: (1) raw milk, (2) evaporated milk (vacuum process), (3) same as (2) but sterilized, (4) evaporated milk made by the aeration process, and (5) same as (4) but sterilized. These milks were fed at 5, 10, 12, 15, and 20 cc. levels, all evaporated milks being diluted and fed in comparison with the raw milk from which they were made. 198 rats were fed the Steenbock yellow corn rachitic diet (supplemented with milk) for 21 days, at which time they were etherized and the femur bones were extracted and ashed. Line tests on bones and weights of thymus glands were recorded also.

It was found (1) that 12 cc. of raw milk were required to prevent a decrease in the ash content of femur bones during a 21 day feeding period, (2) that vitamin D was partially destroyed by vacuum and aeration evaporation, (3) that this destructive effect was enhanced by sterilization, (4) that the aeration method possessed a greater destructive effect than the vacuum process, (5) that while the line test is very useful the bone ash method seems to give a better idea of destruction from the quantitative standpoint, and (6) that the thymus glands have a tendency to hypertrophy on those levels of vitamin D feeding where calcium deposition is faulty.

**THE RELATION OF INORGANIC IRON TO NUTRITIONAL ANEMIA.\***

BY HELEN S. MITCHELL AND MARGERY VAUGHN.

*(From the Nutrition Laboratory, Battle Creek College and Battle Creek Sanitarium, Battle Creek.)*

In a previous publication there was reported a method for producing a true nutritional anemia in young rats by the exclusive feeding of a basal diet of whole cow's milk. At that time there were also reported results on the corrective action of iron from a few sources. These experiments have now been extended, testing out the comparative availability of a larger number of iron compounds. The usual procedure has been to start the anemic rats at weaning on 0.4 mg. of iron from the supplementary source, increasing it at the end of 6 to 8 weeks to 0.8 mg. and again later if the smaller quantity had little or no effect. Hemoglobin determinations were the chief criterion but red cell counts were also made.

The iron salts of organic acids showed widely varying results. Ferrous lactate and ferric tartrate were poorly utilized, while ferric acetate and ferric albuminate were well utilized. So also was ferric ammonium citrate previously reported. Saccharated ferrous carbonate proved to be more easily available than the less soluble ferrous carbonate. Saccharated and peptonized ferric oxide were both more available than the simple ferric oxide with which extremely variable results have been obtained. The soluble modifications of these salts seem to be better utilized for hemoglobin synthesis than the simple salts. Ferrous iodide stimulated prompt and rapid rises in hemoglobin in several cases but failed to maintain the level. Increasing the quantity stimulated a temporary response only. Reduced iron (ferrum reductum, Merck) was poorly utilized and was accompanied by diarrhea in every case. Ferric cacodylate administered orally caused such severe diarrhea and loss of weight that the experiment had to be discontinued.

Judged from the rapidity and height of the hemoglobin response, the iron compounds investigated may be grouped as follows:

\* Presented before the Society of Pharmacology and Experimental Therapeutics.

Good, ferric acetate, ferric albuminate.

Fair, peptonized ferric oxide, saccharated ferric oxide, saccharated ferrous carbonate, ferrous iodide.

Poor, ferrous carbonate, ferric tartrate, ferrous lactate, ferric oxide, ferrum reductum.

#### **BROMINE EXCRETION FOLLOWING BROMOFORM ANESTHESIA.\***

By G. H. W. LUCAS, W. EASSON BROWN, AND V. E. HENDERSON.

*(From the Department of Pharmacology, University of Toronto, Toronto, Canada.)*

The toxicity of chloroform has frequently been ascribed to decomposition of some of the chloroform in the body. Owing to the presence of large amounts of chlorides this change cannot be detected chemically; consequently it was proposed to study bromoform and other brom-alkyl anesthetics. It has been found that there is a decomposition of bromoform, ethyl bromide, and ethylene bromide subsequent to the period of anesthesia; this is indicated by the excretion of bromides in the urine. It is hoped to parallel the amounts of this excretion with the hepatic toxicity of the compounds in question.

#### **THE FATE OF COLLOIDAL LEAD COMPOUNDS AFTER INTRAVENOUS INJECTION.\***

By FRITZ BISCHOFF, N. R. BLATHERWICK, AND ELSIE HILL.

*(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)*

Effects of colloidal lead on oxyhemoglobin.

Effects of colloidal lead on hemolysis.

Toxicity of different lead compounds as determined by (a) death  
(b) effect on hemoglobin.

Effect of addition of lead ions to blood serum and cells.

Excretion of lead.

Retention and distribution of lead in the body.

\* Presented before the Society of Pharmacology and Experimental Therapeutics.

**THE DETERMINATION OF TYROSINE AND TRYPTOPHANE IN PROTEINS.<sup>32</sup>**

By OTTO FOLIN.

**SOME ADDITIONAL OBSERVATIONS UPON THE OVARIAN HORMONE.**

By EDWARD A. DOISY, C. N. JORDAN, AND S. THAYER.

**THE BUFFERING OF THE TISSUES AS INDICATED BY THE CO<sub>2</sub> CAPACITY OF THE BODY.<sup>33</sup>**

By R. J. BROCKLEHURST.

**OXYADENINE.<sup>34</sup>**

By MARY V. BUELL AND MARIE E. PERKINS.

**THE UTILIZATION OF SOME PEPTIDES AND A DIKETOPIPERAZINE CONTAINING CYSTINE, FOR THE NUTRITIVE REQUIREMENTS OF THE WHITE RAT.<sup>35</sup>**

By GEORGE T. LEWIS AND HOWARD B. LEWIS.

**VIII. VITAMIN E POTENCY OF UNSAPONIFIABLE MATTER FROM CRUDE COTTONSEED AND CRUDE CORN OILS.****IX. COD LIVER OIL VERSUS WHEAT OIL AS SOURCES OF VITAMIN E.****X. LACTATION STUDIES ON SYNTHETIC DIETS CONTAINING ALCOHOLIC EXTRACTS OF WHEAT EMBRYO AS THE ONLY SOURCE OF VITAMIN B.****XI. VITAMIN E POTENCY OF BUTTER FAT.**By BARNETT SURE.<sup>35</sup>**II. A BIOLOGICAL METHOD FOR THE STUDY OF THE RÔLE OF VITAMIN B IN LACTATION.**

By BARNETT SURE.

**THE GLYCOGEN CONTENT OF FETAL HEART AND SKELETAL MUSCLE.**

By J. B. COLLIP.

<sup>32</sup> Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, lxxiii, 627.<sup>33</sup> Brocklehurst, R. J., and Henderson, Y., *J. Biol. Chem.*, 1927, lxxii, 665.<sup>34</sup> Buell, M. V., and Perkins, M. E., *J. Biol. Chem.*, 1927, lxxii, 745.<sup>35</sup> Lewis, G. T., and Lewis, H. B., *J. Biol. Chem.*, 1927, lxxiii, 535.<sup>35</sup> Sure, B., *J. Biol. Chem.*, 1927, lxxiv, 37, 45, 55, 71.

**CHEMICAL ANALYSIS OF THE DEGENERATED TESTES OF RATS  
ON A DIET DEFICIENT IN VITAMIN E.**

By H. A. MATTILL.

**THE BIOLOGICAL VALUE OF THE PROTEINS OF MILK AND OF  
CEREAL BREAKFAST FOODS, AS DETERMINED ON RATS.**

By H. A. MATTILL.

**THE ACTION OF NEUTRAL SALTS IN THE HYDROLYSIS OF  
PROTEINS BY PEPSIN.**

By T. L. McMEEKIN.

**STUDIES ON OPTICALLY ACTIVE DYES.**

By FELIX SAUNDERS.

**BIOCHEMICAL STUDIES ON EXTRACTS FROM TESTES.**

By LEMUEL McGEE.

**THE BASAL METABOLISM OF OVERWEIGHT BOYS AND GIRLS  
ELEVEN AND TWELVE YEARS OF AGE.**

By GRACE MacLEOD.

**VITAMIN A IN EVAPORATED MILKS MADE BY VACUUM AND  
AERATION METHODS.**

By R. ADAMS DUTCHER, HANNAH E. HONEYWELL, AND  
C. D. DAHLE.

**THE ASSIMILATION OF THE FAT-SOLUBLE VITAMINS A AND D  
IN THE PRESENCE OF PARAFFIN OIL.**

By R. ADAMS DUTCHER AND J. O. ELY.

**THE OXYGEN ACTIVITY OF THE BLOOD SERUM AND ITS RELATION  
TO THE BASAL METABOLIC RATE.**

By ALFRED E. KOEHLER AND C. W. MATTHEW.

**THE UTILIZATION OF WHEAT BRAN BY THE DOG.**

By GEORGE ALBERT WILLIAMS.

**A QUANTITATIVE METHOD FOR TRYPSIN.**

By OSCAR HELMER.

**STUDIES IN METHEMOGLOBINEMIA.**

By C. C. GUTHRIE, W. S. McELROY, AND T. K. KRUS



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